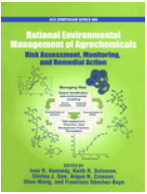

ACS SYMPOSIUM SERIES 966

# Rational Environmental Management of Agrochemicals

## Risk Assessment, Monitoring, and Remedial Action

EDITED BY
Ivan R. Kennedy, Keith R. Solomon,
Shirley J. Gee, Angus N. Crossan,
Shuo Wang, and Francisco Sánchez-Bayo

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# Rational Environmental Management of Agrochemicals

## Risk Assessment, Monitoring, and Remedial Action

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**Sponsored by the**
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American Chemical Society, Washington, DC

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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# Chapter 1

# Achieving Rational Use of Agrochemicals: Environmental Chemistry in Action

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Agrochemicals will remain essential for economic production of the bulk of the world's food and fibre. However, their continued acceptance as agents for the control of pests has required a remarkable evolution in applied chemistry. To guarantee increasing environmental safety, an ongoing process of rational human action is needed, based on chemical risk assessment, chemical monitoring and risk management using information of the highest possible quality as feedback.

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# Introduction

Chemical pesticides of increasing efficacy and selectivity have played an essential role in the production of food and fibre ever since agricultural production moved beyond the stage of local production and consumption into trade. Particularly during the past half-century, it is possible to show an intimate relationship between advances in pesticide chemistry and progress in many other areas of chemistry. Much chemical ingenuity was involved in the discovery and development of these pesticides, often relying on new techniques linking chemical structure to mode of action and efficacy to control pests.

The response in food production to meet the nutritional needs of the earth's increasing human population (five billion in the 20th century), now known as the green revolution, depended critically on the development of several generations of chemical pesticides, not just on the availability of fertilizers such as urea and superphosphate. Pesticides continue to be used as an essential part of the supply chain for both food and fibre for the protection of crops and goods in storage,. The primary reason for their continued use is economic. Benefit-cost analyses consistently show highly significant ratios favoring their application by farmers (*1*). Despite strong public attitudes opposing their use, because of their perceived risks and a significant demand for 'organic' products grown free of agrochemicals (*2*), the vast majority of the world's agricultural production still involves their use and in many regions they are essential maintaining a reliable food supply.

However, the basis for selection and use of these agrochemicals has been subject to continuous evolution. The initial widespread use of organochlorine and organophosphate insecticides was followed by the development of pyrethroids and many other more selective and less persistant chemicals (*3*). Herbicides and fungicides have undergone a similar evolution, responding in part to a market demand for safer chemicals. This evolution has been driven by increased understanding of the environmental fate of agrochemicals, a result of our greater knowledge of their physical, chemical, and biological properties. Improving methods of analysis have been an essential feature of this evolution. More recently, the development of multidimensional mass spectrometric methods coupled to gas and liquid chromatography has provided an even higher level of proficiency in their analysis as chemical residues in produce and in the environment (*4*).

# Regulation of Pesticide Use

An evolution has also occurred in the regulation of the use of agrochemicals. Most countries have developed a legal framework for the registration and use of

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pesticides, requiring the highest standards of information regarding their safe use and efficacy. Companies seeking to register new pesticides must meet stringent requirements for information regarding their effective application and ecotoxicology (*5*). The development period, from first synthesis (discovery) to commercialization, for a new product has increased from 8.3 years in 1995 to 9.1 years in 2000. In the year 2000, it cost an average of US $184 million to discover, develop and register each new crop protection product (*6*). This cost is eight times greater than 20 years earlier and involves extensive laboratory and field work. Apart from the need for information to optimise efficacy and their effective application to crops, this information must allow the environmental fate of each chemical to be predicted. Data on solubility in water and other liquids such as organic solvents and fats expressed as distribution coefficients (see Chapter 2) is a primary requirement.

In addition, an extensive series of tests to determine the ecotoxicology of each chemical is required. A significant number of non-target organisms must be studied to determine the degree of hazard each chemical can present. These will include tests on representative animals, plants and microorganisms.

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## Pacifichem 2005

Several symposia at previous congresses in the Pacifichem series since the 1980s have focussed on the analysis and ecotoxicology of pesticides. For example, in 2000, the theme of one symposium was related to the development and use of immunoassays to measure residues in produce and to study their environmental fate.

In 2005, the symposium was designed to focus on the behaviour in the environment of organic agrochemicals, such as insecticides and herbicides and the development of rational methods aimed at reducing their overall environmental impacts, while promoting their efficacy. The symposium involved three main sessions of oral presentations covering the themes:

i. Risk assessment and modeling, to integrate and relate physical and chemical properties and environmental action, using fugacity modeling and other methods for estimating exposure and risks to the environment and humans, as well as from contamination of food and other consumables.
ii. Improving analytical techniques - with an emphasis on rapid methods derived from biotechnology, such as ELISA and biosensors, to supplement advancing instrumental methods - for assays of produce, hazardous or trade waste, and environmental samples, providing feedback to allow better choices to be made.

Management, remediation, and current environmental policies, stressing the role of risk management of contamination and human exposure, using practical solutions, including genetic technology and bioremediation.

There has been significant progress in these areas of agrochemistry in the past 5-10 years and the book's chapters illustrate these advances and developing methodologies. Lessons learnt during successful research programs of the past decade (e.g. the "Australian cotton story") are also well illustrated and show how well chemistry and biotechnology can serve agriculture, delivering economic and environmental benefits. The aim of the book was to highlight a set of rational principles that could be used to select and manage the best agrochemicals to use in particular scenarios (soils, water, crops, potential ecotoxicology) and to encourage the registration of new chemicals with reduced risk.

While the book that has arisen from the Pacifichem symposium can claim to have addressed these issues in part, gaps remain. The focus in this book is on research methods and it is not a manual of the methods needed to provide information for regulatory purposes. This chapter will outline what has been achieved but also points out in its conclusion part of what remains to be done.

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## The Book's Contents are Environmental

The book largely retains the order of presentation of topics in the symposium with three areas related to risk assessment, monitoring and management. The chapters represent about one-third of the presentations offered at Pacifichem 2005 with all those selected related to environmental issues.

The book commences with a review of modeling methods related to environmental fate of agrochemicals. Fugacity modeling as a rational method is given a highly practical basis by Mackay and Webster. Their treatment in Chapter 2 provides a useful introduction to these methods and how to apply the concept of fugacity or 'escaping tendency' of pesticides from one compartment to another. Even readers needing more sophisticated modeling can use this as a practical introduction with considerable utility, based firmly on principles of classical physical chemistry.

The need for even simpler indicators of hazard or risk for particular agrochemicals is then met in the succeeding chapters. PIRI provides simple risk indicators for water quality; the evaluation of methodologies to estimate hazard or risk from agrochemicals is well illustrated in the field assessments in rice paddies and in a comparative hazard assessment for various chemicals involved in illegal coca production in Colombia. The section concludes with chapters considering challenges regarding geographical variation of soil sorption data and how to account for such variation using the method of rain simulation, producing site-specific data on runoff and infiltration into soil of pesticides.

The section on monitoring commences with a review of modern methods for rapid testing of environmental matrices. Pacifichem 2000 included a symposium in the agrochemicals section that focused on ELISA methods for pesticide monitoring in produce and the environment. The tradition for applying ELISA to measure agrochemicals has been strengthened by 2005 and has even been applied to GIS mapping of DDE residues (*7*) more than 20 years after the use of DDT for control of agricultural pests was banned. But there are clearly major advances in the conception and the realization of new biosensing methods and the potential use of recombinant antibodies for detecting pesticides and antibiotics in environmental samples, covered in the reviews and case studies given in Chapters 10-13.

Monitoring the risk of exposure to agrochemicals in the human environment is considered in Chapter 14, as well as tools to monitor the risk of environmental exposure to pesticides in Vietnam and Thailand in the succeeding two chapters. The effectiveness of the major investments needed in chemical technology transfer from advanced countries to developing nations is also illustrated by these contributions. To support these outcomes, chemical education is an ongoing process that requires a costly infrastructure for any significant monitoring and management of chemicals. However, this investment is essential if environmental health and high quality produce for international trade are to be achieved.

Since 2000, methods for ELISA of agrochemicals have been extended to the measurement of *Bacillus thuringinesis* toxins in genetically modified (GM) crops, illustrated in Chapter 17. GM technology on agrochemical use is also related in the concluding three chapters of the book, which document the impact of genetically modified cotton on reducing environmental impacts of agrochemicals used to control pests in Australia.

The use of bioremediation enzymes for decontaminating waste water is also an approach to risk management that relies on modern techniques of molecular biology. Chapter 18 reviews the possible role of bioremediation enzymes to degrade carbamate pesticides for reducing environmental risks. This choice illustrates very well the chemical logic involved, because carbamates include pesticides in the three main groups of insecticides, herbicides and fungicides, and effective molecular technology is likely to yield benefits for agrochemicals used for all three purposes. Another multi-purpose area arising from research designed for mass generation of antibodies for ELISA monitoring purposes is the potential to replace fungicides by recombinant antibody technology of plants, as illustrated in Chapter 19; this shows how ingenuity in biological chemistry can manage risk from agrochemicals.

The book concludes with several chapters documenting major success stories related to risk management from agrochemicals in the Australian cotton

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industry. The profile of agrochemicals applied in most countries now for cotton production has been drastically modified by the introduction of GM cultivars of cotton. Control of *Helicoverpa* species by the insertion of toxin genes of the soil bacterium *Bacillus thuringiensis* into cotton and other plants has substantially reduced the need to apply insecticides acting on such chewing insects. Chapter 20 illustrates how environmental risk from agrochemicals has been reduced by the introduction of Bt cotton, as well as by the introduction of herbicide resistance genes into cotton, allowing the crop to be sprayed by environmentally safer herbicides such as glyphosate. As a fibre crop, GM technology for cotton has been met with less resistance by the 'green' political lobby and the uptake of multi-gene GM cotton in Australia (Bollgard© and Roundup Ready©) and many other countries is now almost complete.

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## Environmental Chemistry and Participatory Action

The role of chemistry in society is no longer restricted to research and development conducted behind closed doors, with the finished products eventually being released for sale to markets. The final two chapters in this book illustrate how important it is to involve stakeholders in society in enviroimmental chemical research, particularly at the stage of application. Some salutary lessons learned regarding effective extension of effective agrochemical management practices to farmers are illustrated for the cotton and sugarcane industries. Without involvement by the stakeholders, it is doubtful that the practices recommended could ever be accepted. The links between theory and practice must be made as clear as possible. The final chapter summarises the overall strategy that was adopted to solve environmental problems in the cotton industry, funding the research and generation of best management practices needed to save a productive industry from being closed down because of unacceptable environment practices. All countries can learn from these experiences, that also show how important it is to devote adequate resources to solving the problem.

The strategy for action is really only the application of the scientific method, but at a global scale. Hypotheses are proposed about the environmental fate of agrochemicals, based on their known physicochemical properties and the environmental conditions. Risk assessment requires such information as well as the knowledge of the ecotoxicity of the chemicals involved so that the relationship between exposure and probable effects can be defined. These hypotheses are then tested by effective monitoring of the environment. The results can be fed back into the models for the purposes of validation, if necessary leading to their modification.

Further hypotheses about the best means to manage the risk must then be developed. Some risk management strategies are suggested in this book, but it is important that they be designed with both environmental and economic benefits in mind. Clearly, management has a strong economic dimension and benefit-cost analysis must be applied to the management practices or risk reduction to have any chance of being sustainable. Farmers especially will not apply practices that cost more to implement than the perceived economic benefits they generate.

## Regulatory procedures

An important area that could not be included in this book is improving the chemical technology needed for the purposes of regulation. The application of agrochemicals is increasingly controlled by legal means, usually by instructions on the product label. Most governments have set up national agencies to regulate the application of agrochemicals and environmental protection authorities to advise these agencies and to monitor their use (*8*). National residue surveys paid for by industry monitor the quality of food and its freedom from chemical residues above an maximum residue level (MRL) providing a trigger point for action to improve practices.

The registration for use of a new agrochemical is a very costly process. Regulatory agencies require the submission of extensive data sets regarding the physical and chemical properties of chemicals, relating to efficacy as pesticides and to the conditions for application in the field. This is the same information needed for fugacity modeling. Extensive information regarding their ecotoxicity is also required, as well as laboratory and field studies confirming environmental behaviour. Although there is some discussion of these issues in this book, there is a need to develop more flexible rational protocols and the use of environmental models that more accurately predict potential risk. The agrochemical industry must have clear guidelines for the purpose of generating this information as it is the basis for rational decision making.

The effective regulation of the registration and use of agrochemicals is a challenging task. However, because of its importance for agricultural productivity and human and environmental health, the highest standards of quality control should be applied to the process of decision making for regulation. The quality of scientific peer review and analysis applied in the regulatory agencies needs to be very high. Unless this area is properly resourced with scientific personnel having sufficient expertise, effective regulation will be impossible.

Some features of regulatory systems that could help guarantee improved standards are:

- Transparency or visibility in operations
- Independent peer review of documentation

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- Objective science free of political expediency
- Willingness to accept criticism and to excel in response

Other desirable features that may require law reform are:

- More certainty in the requirements for the process
- Benefit-cost analyses of recommended changes, for both environmental values and agricultural productivity
- Acknowledgement of site specificity and of the appropriateness of the use of regional approaches such as watershed assessment.

The result could then be regulatory decision making that would meet world best standards, winning the confidence of industry and the community.

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## Monitoring and feedback

Environmental monitoring is essential, even if this is only possible for some situations. As the final chapter illustrates, it is possible to effectively 'clean-up' riverine systems, but this will only be confirmed if costly monitoring programs are undertaken. Without such knowledge we are merely guessing that any improvement has occurred. However, the knowledge that endosulfan, a chemical highly toxic to fish and other species, is no longer a serious contaminant in certain Australian rivers has considerable economic value. This is not only in avoiding even more costly regulation but also in terms of increased biological productivity of riverine systems.

Monitoring should be highly targeted, to reduce its cost. Estimates of inputs of agrochemicals into agriculture are also important, so that the scale of the hazard can be estimated. Decisions regarding monitoring should clearly be based on such information. There is a role here for geographical information systems (GIS), in establishing the pattern of contamination (*7*). Historical data are also important, since by correlation, it can indicate the importance of environmental factors such as the weather, indicated in Chapter 20.

## Cooperative participatory action

Action is a quantity with the physical dimensions of angular momentum or spin (*9*). It is the product of *energy* or *work* by *time*. All of the chemical activities implied by the chapters of this book imply action for their meaning to be expressed.

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Participatory action involves a rational or scientific approach to human activity where, as the process concerned unfolds, the participants respond to emerging information generated by the process. It infers an ongoing process in which decisions are made as logical choices based on various monitoring tests. Response curves can provide sensitivity analysis, indicating if the investment of resources needed to effect improvements is justified. The optimum state is achieved as a result of small adjustments in the system in response to this feedback.

A feature of participatory action is the power of timely cooperation to reduce the need for energy inputs. Less energy is needed to sustain cooperative systems and the greater momentum of the larger aggregates delivers more force. The increased power of cooperative activity has to be balanced against the tendency for inertia to increase as mass and size of functional units increases. Achieving such efficiency in the application of chemistry to society to minimize risks such as those posed by agrochenicals and to maximize their benefits is also an exercise in participatory action.

## Conclusion

It is at the stage of risk management that the interaction between the available technology and social action reaches its greatest potential. For example, the introduction of GM crops can involve a large change in human behaviour and agricultural practice. Consultation with stakeholders is an important feature of such processes, as illustrated by the conclusions in the final Chapter. Indeed, the complex network of participants in such technology transfer requires cooperativity on an extraordinary scale.

We conclude this chapter by summarizing the overall aim of the book. Figure 1 provides an outline of the three main stages of risk assessment, monitoring and risk management represented in the chapters of this book.

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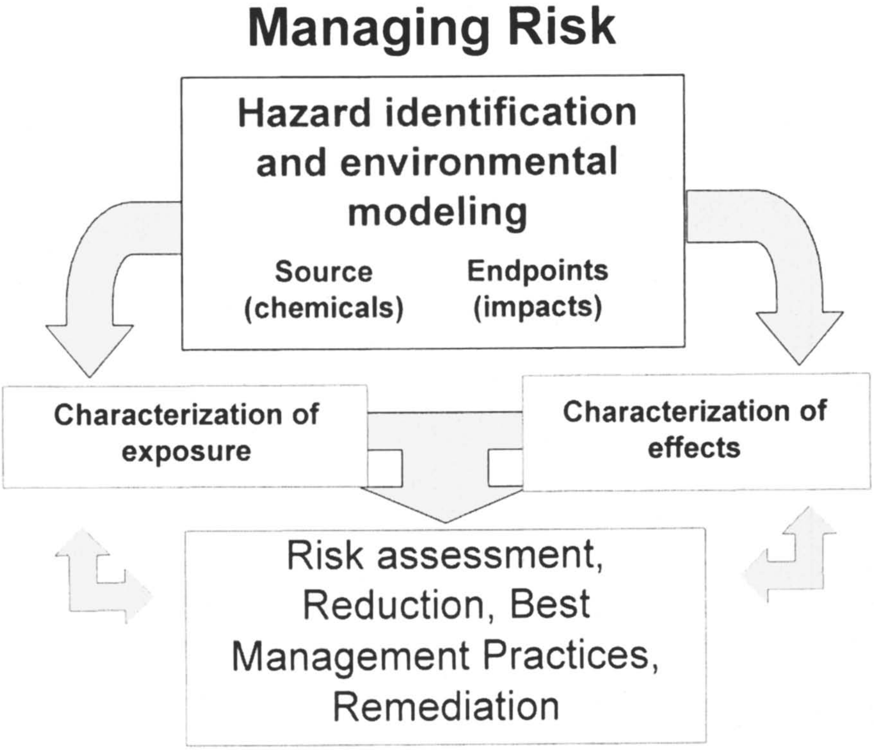
Managing Risk
Hazard identification and environmental modeling
Source (chemicals)
Endpoints (impacts)
Characterization of exposure
Characterization of effects
Risk assessment, Reduction, Best Management Practices, Remediation

*Figure 1: Scheme for risk assessment, monitoring and risk management for agrochemicals. The role of feedback is emphasized.*

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## Chapter 2

# Simple Fugacity Models of Off-Site Exposure to Agrochemicals

**Don Mackay and Eva Webster**

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A brief review is presented of the environmental and social incentives for quantifying the fate of agrochemicals and for using mass balance models to complement empirical measurements of concentrations resulting from specific applications. The role of the fugacity concept for use in such models is described and discussed. It is suggested that four relatively simple fugacity models can play a useful role in these agricultural situations by quantifying partitioning, loss processes (especially degradation) and transport processes in bulk soil, vegetation, invertebrates, and small mammals at a screening level. In total, these models provide information not only on the fate of pesticides and other "inert" ingredients in the soil but also on the potential for contamination by off-site transport. Their use can, we suggest, contribute to more effective selection and application of these agrochemicals, to reduced risk to humans and wildlife, and ultimately to increased public acceptance of their benefits.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
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## Introduction

Whereas there is widespread public acceptance that pesticides play an invaluable role in agriculture and can be safely applied, there is increasing concern that there may be public exposure "off-site". Examples include pesticides used for non-agricultural purposes such as indoors, for weed or rodent control in urban areas, as residues in and on foodstuffs, and contamination of nearby water bodies by run-off and atmospheric transport. Whereas the public generally accepts the merits of using pesticides to help control unwanted organisms and plants in the vicinity of applications, they are often intolerant of the presence of these substances "off-site" where exposure is inadvertent and non-target organisms may be affected. This intolerance translates into political pressure and ultimately can result in regulatory actions to minimize or eliminate off-site or far-field exposure. In Canada, many municipalities have responded to such concerns by introducing bans on the "cosmetic" use of pesticides. The public has shown a willingness to pay a premium for "organically grown" or pesticide-free foods. Given this growing intolerance of off-site exposure there is an increased incentive to ensure that pesticides are used safely and sustainably and with a full knowledge of their fate. The same concern applies to metabolites or degradation products and to other "inert" ingredients of the formulations such as carriers, solvents, adjuvants, surfactants, and impurities. Environmental science can respond to these concerns in two ways. First is to enhance our ability to quantify the fraction of the pesticide applied that is not degraded at the application site and may be transported to other locations, possibly resulting in human or wildlife exposure. This information must be provided in a simple, transparent, and credible manner if it is to be accepted. It can assist in selecting pesticides that will have less off-site impact. Second, and more difficult, is to estimate the nature and magnitude of adverse effects or toxicity of the often low doses or exposures. Here we address only the first task.

The primary and most convincing method of determining quantities experiencing off-site transport is monitoring programs in which samples of air, water, soils, and biota including produce are taken at various time intervals both on-site and off-site following application and analysed for residues and possibly metabolites. This is a difficult, demanding, and expensive task. It may be that the results are significantly affected by prevailing weather conditions, notably temperature, wind, and precipitation events. Given these difficulties and the need to extrapolate the results to other application conditions, there is an incentive to use mass balance models to reconcile the empirical observations and deduce how a given substance will behave under a variety of conditions.

Mass balance models are widely and successfully used to describe the fate of pesticides applied for agricultural purposes. Examples include PRZM *(1)*, CREAMS *(2)*, GLEAMS *(3)*, and BAM *(4)*. The use of models has become

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sufficiently accepted that government agencies such as CREM (Council for Regulatory Environmental Modeling, under the US EPA) have been set up to provide guidance on the selection and use of models for regulatory purposes *(5)*. These models can be used in a predictive mode but more commonly they are used in conjunction with monitoring data to provide a complete picture of the fate of the active ingredient, and possibly of its degradation products. An incentive for applying models is that it is not possible to make direct measurements of the rates of certain processes in the field. It is only possible to measure concentrations at various places and times and from these data infer process rates. For example, an observed decrease in concentration may be attributed to degradation, volatilization or leaching, thus the individual rates of these processes may not be determined unambiguously in a single field experiment; only their total rate can be determined. These individual rates can, however, be estimated using a reliable model that has been subjected to case-specific validation for a number of chemicals. The model thus serves the fundamentally important purpose of synthesizing the available information into a coherent statement of mass balance. A successful statement implies a sufficiently complete understanding of the system. Public perceptions and sound regulatory policies should be based on such an understanding.

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## Far-field or Off-site Transport and Exposure

Far-field exposure can result from the substance being present in a variety of environmental media.

- Volatile substances can be transported in air flow from the application site becoming dispersed over large areas. The chemical may degrade, be inhaled or be deposited by wet and dry deposition to soils, vegetation and water bodies.
- Water soluble substances may leach into groundwater or run-off to surface waters. There may then be contamination of aquifers and wells and there may be impacts on organisms in ditches, rivers and lakes.
- Soil particles may be transported from the site, along with associated chemical in air-borne or water-borne solids.
- Biota ranging from insects to amphibians, birds and mammals may become contaminated in the near-field and migrate to the far-field. As prey, they may introduce the pesticide into the food webs. Some fish- and insect-eating birds appear to be particularly vulnerable.
- Finally, agricultural produce may convey quantities of the chemical to the far-field for ultimate consumption by humans or other animals.

A process that is not considered here is off-site transport during application by spray drift. Transported quantities may be appreciable, but they depend on the application process.

We suggest that it is useful to have available a set of relatively simple but comprehensive models that can be used to assess which, if any, of these processes may be significant. If a specific transport process is judged to be significant, a more detailed model can be applied. Examples are models that address specific aspects of pesticide fate such as migration through the soil column, run-off, evaporation followed by atmospheric dispersion, uptake in growing vegetation, or bioaccumulation in birds. In this paper we describe simple models and present illustrative results for a number of compounds. It is hoped that the models may be of value for assigning order-of-magnitude estimates to fate processes and thus direct priorities for subsequent model applications and monitoring programs. Far-field exposure assessment is thus addressed as a two-stage process: simple or screening-level followed by detailed process-specific models.

We further suggest that fugacity modeling is particularly suitable for screening level assessments. It is also suitable for certain detailed assessments, especially in defined environments such as indoors and when the pesticide is relatively persistent. When degradation rates are fast, many of the equilibrium assumptions inherent in fugacity models break down and conventional concentration-kinetic models may be preferred. However, it is the more persistent substances that have a potential for far-field effects and are generally of greatest concern.

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## The Fugacity Concept

Although most models are written in concentration format, in many respects fugacity models are advantageous for simple models of the type described here. In principle, both concentration and fugacity models will give identical results when provided with identical input parameters and fate equations. They are thus fully equivalent and inter-convertible.

The concept of fugacity was introduced over a century ago by G.N. Lewis *(6)* as a criterion of equilibrium. It can be viewed as a partial pressure or escaping tendency and has units of pressure (Pa). When a chemical achieves equilibrium between different phases such as pore air, pore water, organic matter and a plant root, the fugacities in each phase are equal. The concentrations differ, however, often by many orders of magnitude. They are related by partition coefficients such as $K_{AW}$ the air-water partition coefficient. In the fugacity formalism each phase is assigned a capacity term Z, the proportionality constant between concentration and fugacity, f , namely C (mol/m$^3$) = Z (mol/m$^3$Pa) . f (Pa). Clearly, the air-water partition coefficient $K_{AW}$ is simply the ratio of the Z values in air and water, i.e., $K_{AW} = C_A/C_W = Z_Af/Z_Wf = Z_A/Z_W$. Essentially, a Z value is "half" a partition coefficient. Figure 1 gives an example of an equilibrium distribution calculation of a defined quantity of chemical between four soil phases. The mass balance equation states that the total amount of chemical, M (mol) must equal the sum of the amounts in each phase $m_i$ (mol) which in turn must be $V_iC_i$ or $V_iZ_if$ where $V_i$ is the phase volume (m$^3$), $C_i$ the concentration (mol/m$^3$), $Z_i$ is the phase specific capacity term, and f is the common fugacity. Specifically, $M = \Sigma m_i = \Sigma V_iC_i = \Sigma V_iZ_i f_i = f\Sigma V_iZ_i$ thus $f = M/\Sigma V_iZ_i$. From f, all the concentrations can be calculated as $fZ_i$ and the masses as $fV_i Z_i$.

It is noteworthy that all concentrations are expressed on a volumetric basis rather than on a mass basis as is usual for solid phases. The phase density is required for conversion between these concentrations.

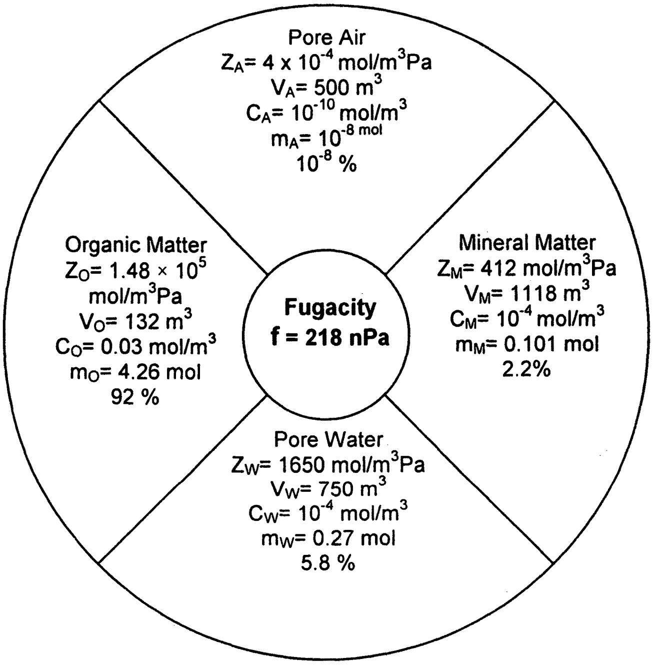
Pore Air
$Z_A$= 4 x 10$^{-4}$ mol/m$^3$Pa
$V_A$= 500 m$^3$
$C_A$= 10$^{-10}$ mol/m$^3$
$m_A$= 10$^{-8}$ mol
10$^{-8}$ %
Organic Matter
$Z_O$= 1.48 × 10$^5$ mol/m$^3$Pa
$V_O$= 132 m$^3$
$C_O$= 0.03 mol/m$^3$
$m_O$= 4.26 mol
92 %
Fugacity
f = 218 nPa
Mineral Matter
$Z_M$= 412 mol/m$^3$Pa
$V_M$= 1118 m$^3$
$C_M$= 10$^{-4}$ mol/m$^3$
$m_M$= 0.101 mol
2.2%
Pore Water
$Z_W$= 1650 mol/m$^3$Pa
$V_W$= 750 m$^3$
$C_W$= 10$^{-4}$ mol/m$^3$
$m_W$= 0.27 mol
5.8 %

*Figure 1. Equilibrium distribution of atrazine between the four soil phases as calculated by Model 1. The partition coefficient for any pair of phases is the ratio of their Z values, e.g., $K_{AW} = Z_A/Z_W = 4 \times 10^{-4} / 1650 = 2.4 \times 10^{-7}$. The concentration in any phase is the the fZ product, e.g., $218 \times (1.48 \times 10^5)$or 0.03 mol/m$^3$ for organic matter.*

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The Z values depend on the phase and on chemical properties such as vapor pressure, solubility in water, the octanol-water partition coefficient, $K_{OW}$, and the organic carbon-water partition coefficient, $K_{OC}$. They are thus temperature dependent. If sorption is linear, Z values are constant, but under non-linear conditions they vary with concentration and Freundlich or Langmuir expressions must be included. Under the dilute conditions normally found in the environment Z values are constant at an "infinite dilution" value. Thus Z is first defined for the air phase, using the Ideal Gas Law, as 1/RT where R is the gas constant and T is absolute temperature. For other phases Z can be calculated from measured or estimated partition coefficients. Definitions are summarised in Table I, full details and justification are given by Mackay *(7)*.

**Table I. Definitions for fugacity capacities, $Z_i$ as described in Mackay *(7)* except $Z_Q$ which from Arnot et al *(16)*.**

| | | |
|---|---|---|
| Air | Vapor | $Z_A = 1 / (RT)$ |
| | Aerosols | $Z_Q = 0.1\ K_{OA}\ Z_A$ |
| | Bulk | $Z_{Air} = Z_A\ \phi_{Vapor} + Z_Q\ \phi_Q$ |
| Water | | $Z_W = WS \cdot MW / VP$ |
| Soil | Pore Air | $Z_{PoreAir} = Z_A$ |
| | Pore Water | $Z_{PoreWater} = Z_W$ |
| | Organic Matter | $Z_{OM} = K_{OM}\ Z_W$ |
| | Mineral Matter | $Z_{MM} = K_{MW}\ Z_W$ |
| | Bulk | $Z_E = Z_{PoreAir}\ \phi_{PoreAir} + Z_{PoreWater}\ \phi_{PoreWater} + Z_{OM}\ \phi_{OM} + Z_{MM}\ \phi_{MM}$ |
| Flora | Roots | $Z_{Rt} = x_{Rt}\ K_{OW}\ Z_W$ |
| | Above-ground vegetation | $Z_{Sht} = x_{Sht}\ K_{OW}\ Z_W$ |
| Fauna | Invertebrates | $Z_{Worm} = x_{Worm}\ K_{OW}\ Z_W$ |
| | Small terrestrial animals | $Z_{Shw} = x_{Shw}\ K_{OW}\ Z_W$ |

where R = gas constant
T = air temperature, K
$\phi_i$ = volume fraction of phase i in the bulk medium
WS = water solubility, $g/m^3$
MW = molar mass, g/mol
VP = vapor pressure, Pa
$x_i$ = octanol-equivalent fraction

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and subscripts are

A = air vapor
W = water
Q = aerosols
OM = organic matter in soil
MM = mineral matter in soil
E = soil, or "earth"
Rt = roots
Sht = above-ground vegetation, or "shoots"
Shw = small terrestrial animals, or "shrews"

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Equilibrium distributions are thus easily deduced, but usually conditions are non-equilibrium and it is necessary to estimate transport and transformation rates. When mass balance equations are written to describe these non-equilibrium conditions in terms of fugacity the rate, N (mol/h), is given by Df where D is a fugacity-based rate constant with units of mol/Pa.h. D values can be defined for a variety of processes including degrading reactions, evaporation, leaching, uptake by roots from soil, and feeding by organisms. Table II summarises the definitions of D values used in this context as a function of kinetic terms including degradation rate constants, mass transfer coefficients and flow rates.

D values are essentially conductivities, thus a large D value indicates a fast process. When loss processes from a phase occur in parallel the D values add. For series resistance processes the resistances, or reciprocal D values, add. This commonality of transport and transformation rate coefficients is very convenient in that they can be compared and added. This is not possible with concentration-based parameters and is therefore one of the great strengths of the fugacity concept.

## Chemical Properties

Five chemicals, including pesticides and solvents, namely Atrazine (Atr), Benzene (Ben), Diazinon (Dia), DDT, Pendimethalin (Pen), and Pyrene (Pyr), were selected to cover a wide range of partitioning properties. Table III lists the properties of these chemicals estimated from the literature. These have been chosen. This range is conveniently displayed by locating the chemicals on a "chemical space" diagram as a plot of log $K_{AW}$ vs log $K_{OW}$, illustrated in Figure 2.

Table II. D values as functions of kinetic terms including degradation rate constants, mass transfer coefficients and flow rates as discussed in Mackay *(7)* except where noted. Diffusion is based on the Millington-Quirk equation. Parameter values and common symbols are given in Table IV.

| | | | |
|---|---|---|---|
| Degradation in Soil | | | $D_{React} = V_E Z_E k_{React}$ |
| Leaching | | | $D_L = G_L Z_W$ where $G_L = LR A_E / 1000 / 24$ |
| Volatilization from soil | | | $D_V = 1 / (1 / D_E + 1 / (D_{SA} + D_{SW}))$ |
| | Air boundary layer | | $D_E = Area K_{EV} Z_A$ |
| | Diffusion | in air | $D_{SA} = A_E DIFEA Z_A / YD$ |
| | | in water | $D_{SW} = A_E DIFEW Z_W / YD$ |
| | Mass transfer coefficient | | $K_{EV} = DIFMA / THICK$ |
| | Effective diffusivity | air | $DIFEA = Q_A MDA / 24$ |
| | | water | $DIFEW = Q_W MDW / 24$ |
| | Porosity | air | $Q_A = \phi_{PoreAir}{}^{(10/3)} / (\phi_{PoreAir} + \phi_{PoreWater})^2$ |
| | | water | $Q_W = \phi_{PoreWater}{}^{(10/3)} / (\phi_{PoreAir} + \phi_{PoreWater})^2$ |
| Air Advection | | | $D_{AA} = (V_A Z_A + V_Q Z_Q) / ResTime$ |
| | | | $ResTime = (\sqrt{}A_E)/(3600 Wind)$ |
| Net volatilization | | | $D_{VO} = 1/ (1/D_V + 1/D_{AA})$ |
| Deposition from air | | | $D_{AirE} = D_{Rain} + D_{Q\,Dry} + D_{Q\,Wet} + D_V$ |
| | Rain (based on (16)) | | $D_{Rain} = A_E * Rain * Z_W$ for $K_{AW} \geq 1/S$ |
| | | | $D_{Rain} = A_E * Rain * S * Z_A$ for $K_{AW} < 1/S$ |
| | Aerosol deposition | dry | $D_{Q\,Dry} = A_E \phi_Q DryDep Z_Q$ |
| | | wet | $D_{Q\,Wet} = A_E \phi_Q Rain Z_Q S$ |
| Exchange | Air-shoot | | $D_{AirSht} = V_{Sht} k_{Sht} Z_{Air}$ and $D_{ShtAir} = D_{AirSht}$ |
| | Soil-root | | $D_{ERt} = V_{Rt} k_{Rt} Z_{PoreWater}$ and $D_{RtE} = D_{ERt}$ |
| | Root-shoot | | $D_{RtSht} = D_{RtE}$ and $D_{ShtRt} = D_{RtSht}$ |
| | Soil-worm | | $D_{EWorm} = V_{Worm} k_{Worm} Z_E$ and $D_{WormE} = D_{EWorm}$ |
| Animal uptake from invertebrates | | | $D_{WormShw} = V_{Shw} k_{Shw} Z_{Worm}$ |
| Animal loss to soil | | | $D_{ShwE} = D_{WormShw} / BMF$ |

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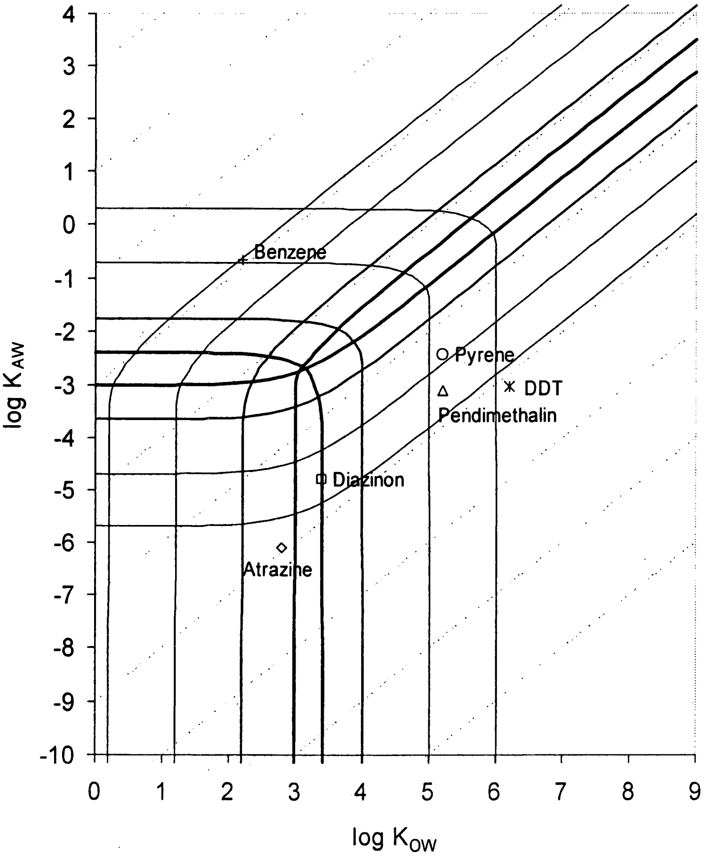

*Figure 2. Selected substances are related to the range of chemical space of a log $K_{AW}$ vs log $K_{OW}$ plot. The diagonal lines represent constant values of log $K_{OA}$ ($K_{OA} = K_{OW}/K_{AW}$) and the curves represent constant partitioning to the phase; air, water, or octanol (or octanol-equivalent fraction). The intersection of the thickest curves represents the case of a substance partitioning equally to all three phases. The thickness of these curves is reduced as partitioning to the phase is reduced.*

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**Table III. Physical chemical properties of selected substances. Data are taken from Mackay and Stiver *(17)*, Mackay et al *(18)*, Hornsby et al *(19)*, Worthing and Walker *(20)*, and the EPI Suite software *(21)* and assuming $K_{MM} = 0.1$ for all substances.**

| | *Molar Mass g/mol* | *Water Solubility g/m³* | *Vapour Pressure Pa* | *$K_{OC}$ L/kg* | *log $K_{OW}$* | *Half-life in Soil h* |
|---|---|---|---|---|---|---|
| Atr | 215.7 | 32 | $9.2 \times 10^{-5}$ | 160 | 2.75 | 1700 |
| Ben | 78.1 | 1780 | 12700 | 83 | 2.13 | 1200 |
| Dia | 304.4 | 60 | 0.008 | 820 | 3.3 | 1700 |
| DDT | 354.5 | 0.0031 | $2 \times 10^{-5}$ | 240000 | 6.19 | 92000 |
| Pen | 281.3 | 0.59 | 0.004 | 65000 | 5.2 | 1440 |
| Pyr | 202.3 | 0.132 | 0.0006 | 62000 | 5.18 | 17000 |

## Nature and Scope of the Models

In the following sections we describe and apply a series of four simple fugacity models of increasing scope, complexity, and detail. It is suggested that they be applied in sequence.

The first model describes the static equilibrium partitioning of a defined quantity of chemical in the soil. It thus addresses the question of how this chemical will partition between the four soil phases and what are the likely concentrations. The fugacity in the air directly above the soil (referred to as "canopy air") is calculated as a fraction of the fugacity in soil determined by the ratio of the net volatilization D value to the D value for advection in the air. The vegetation and soil fauna are assumed to be at equilibrium with the soil. This simple assumption is likely to over-predict the concentrations in the biota.

The second model includes the canopy air, and vegetation and soil fauna, but allowance is made for the likely failure of the biotic media to achieve equilibrium because of growth dilution and possible biotransformation in the biota. It thus addresses the question of how partitioning may be affected by the presence of biota and the approximate expected biotic concentrations.

The third model includes estimates of the initial rates of loss by reaction and advection processes immediately after application of a defined quantity of chemical. These rates can be used to estimate half-lives. This shows which loss processes are most important and it provides a first estimate of the pesticide's persistence. The fourth model is a dynamic description of the chemical masses in the system as the substance reacts and advects over a period of time.

These models will be available from the authors through the CEMC website (http://www.trentu.ca/cemc). The parameters describing the environment, the chemical properties and application quantities can all be selected by the user.

A series of illustrative applications follows for the environment specified in Table IV. This illustrative environment consists of an area of 1 ha of soil consisting of soil solids (mineral and organic matter), pore water and air. In addition there is an overlying air phase of height 1 m, vegetation and fauna. Z values of these media are defined from the chemical properties in Table III and using the expressions in Table I. If empirical data on soil sorption or air-water partitioning are available they can be readily and directly used to deduce the Z values. It may also prove to be useful to calculate Z for aerosol particles to which evaporated chemical may sorb.

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## Model 1. Static Equilibrium in Soil

This model performs a simple equilibrium distribution calculation. Using the environmental data from Table IV, the chemical property data in Table III, and assuming a mass of chemical, the equilibrium distributions of the chemicals can be estimated for the four soil phases as shown Table V.

This simple equilibrium calculation shows how a given mass of chemical of say 1 kg, will partition at equilibrium between the soil phases, air above the soil ("canopy air"), vegetation, and soil fauna. No degradation kinetics are considered. Of interest are the relative concentrations and masses in each compartment. It is recognized that instantaneous equilibrium will not necessarily be reached, thus the masses and concentrations should be viewed as only indicative of maximum potential values.

Although the percentages in the soil pore water and air are low for all of the chemicals considered here, they display considerable variability reflecting the chemical properties. Table III suggests that a "volatile" chemical is one which has a percentage in air exceeding 0.5 to 1%. Likewise a "water-soluble" chemical has a percentage in water exceeding 0.5 to 1%. These chemicals with low binding to organic or mineral matter are viewed as being particularly susceptible to loss by evaporation or leaching respectively. Chemical such as DDT that tend to bioaccumulate can also be identified by their log $K_{OW}$ exceeding approximately 5. The primary advantage of this calculation is its simplicity and transparency. There is now a quantitative appreciation of the likely partitioning tendency of the substance.

## Non-attainment of Equilibrium

In screening-level models it is often convenient to assume that phases that are in close contact achieve the same fugacity as was done in Model 1. This

**Table IV. Properties of near-field environment as used in this illustration. Values are based on the Soil Model described by Mackay *(7)* except where noted. Symbols used in Table II are given here.**

| *Soil Properties* | | | |
|---|---|---|---|
| Area | | $A_E$ | 1 ha or 10000 $m^2$ |
| Soil depth | | | 0.25 m |
| Diffusion path length | | YD | 0.1 m |
| Volume fraction of aerosols in | | $\phi_Q$ | $2 \times 10^{-11}$ |
| Water leaching rate | | LR | 2 mm/d |
| Air boundary layer thickness | | THICK | 0.00475 m |
| Molecular diffusivity in air | | MDA | 0.43 $m^2/d$ or $4.98 \times 10^{-2}$ $cm^2/s$ |
| Mass fraction of OC in dry soil | | | 0.03 |
| Volume fraction | Pore air | $\phi_{PoreAir}$ | 0.2 |
| Densities | Organic Matter | | 1200 $kg/m^3$ |
| | Mineral Matter | | 2500 $kg/m^3$ |

| *Canopy Air Properties* | | |
|---|---|---|
| Air height | | 1 m |
| Wind speed | Wind | 1 m/s |
| Aerosol scavenging ratio | | 200000 |
| Rain rate | Rain | 0.0001 m/h [a] |
| Aerosol dry deposition rate | DryDep | 10 m/h [b] |

| *Biota Properties* | *Number of Biota per Unit Area $m^{-2}$* | *Volume of Single Organism $cm^3$* | *Octanol-equivalent fractions $x_i$* |
|---|---|---|---|
| Roots | 2 | 100 | 0.05 |
| Shoots | n/a | n/a | 0.01 |
| Invertebrates | 100 [c] | 2 | 0.012 [d] |
| Small animals | 0.01 | 10 | 0.07 [e] |
| Shoot-root volume ratio | 1 | | |
| Density | 1200 $kg/m^3$ | | |
| Uptake rate constants, $h^{-1}$ | | | |
| Air-to-shoots [f] | $k_{Sht}$ | $5 \times 10^5/24$ | |
| Soil-to-roots [g] | $k_{Rt}$ | 38434 | |

*Continued on next page.*

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**Table IV.** ***Continued.***

| | | |
|---|---|---|
| Soil-to-worm [h] | $k_{Worm}$ | ln(2)/120 |
| Worm-to-mammal [i] | $k_{Shw}$ | 38375 |
| Shrew biomagnification factor | BMF | 5 |
| Growth rate as a fraction of body volume per day (for all biota: roots, shoots, worms, and shrews) | | 0.01 $m^3/m^3d$ |

*a* based on *(24)*; *b* based on *(25)*; *c* based on *(22)* for worms; *d* based on *(23)* for worms; *e* based on *(23)* for shrews; *f* based on *(11)*; *g* based on *(12)*; *h* based on *(14)*; *i* based on *(15)*

**Table V. The relative amounts (%) of the selected substances in the four phases in soil as calculated by Model 1.**

| | *Pore Air* | *Pore Water* | *Organic Matter* | *Mineral Matter* |
|---|---|---|---|---|
| Atr | < 0.01 | 5.84 | 91.98 | 2.18 |
| Ben | 1.54 | 10.31 | 84.29 | 3.84 |
| Dia | < 0.01 | 1.21 | 98.33 | 0.45 |
| DDT | < 0.01 | < 0.01 | 99.99 | < 0.01 |
| Pen | < 0.01 | 0.016 | 99.98 | < 0.01 |
| Pyr | < 0.01 | 0.016 | 99.98 | < 0.01 |

assumption avoids the need to calculate rates of intermedia transport or degradation. In some cases this assumption introduces considerable error as is illustrated by the following example. We consider a relatively small phase such as a plant or animal that receives contaminant from its environment by diffusion or mass transport as shown in Figure 3.

$D_{L2}$ includes growth dilution and degradation reactions. $D_{12}$ and $D_{21}$ express diffusive exchange and are assumed to be equal in value. A mass balance at steady-state gives $f_1D_{12} = f_2(D_{21}+D_{L2})$ or $f_2/f_1 = D_{12} / (D_{21}+D_{L2})$ If $D_{12} = D_{21}$ this can be rearranged to give $f_2/f_1 = 1/(1 + D_{L2}/D_{12})$ Further, if $D_{L2} << D_{12}$ then $f_2$ is approximately equal to $f_1$ and the rate of loss from 2 is insufficient to cause a significant fugacity difference. When $D_{L2}$ is approximately equal to $D_{12}$ then $f_2$ is about half $f_1$. When $D_{L2} >> D_{12}$ then $f_2 << f_1$ because of the rapid loss relative to the transfer rate. The fugacity $f_2$ is controlled by the relative kinetics of uptake and loss.

Expressing the D values as GZ products or $D_{L2}$ as a VZk product gives $D_{L2}/D_{12} = G_{L2}Z_2/G_{12}Z_1$ or $V_2Z_2k_{L2} /G_{12}Z_1$. The departure from equilibrium $(f_2/f_1)$ is thus controlled by two ratios. The ratio $Z_2/Z_1$ is $K_{21}$, the equilibrium partition coefficient which varies greatly from chemical to chemical and for hydrophobic substances is also dependent on L, the lipid or octanol-equivalent content of the phase. It can thus be postulated that if phase 1 is air or water, this ratio is L $K_{OA}$ or L $K_{OW}$ respectively where $K_{OA}$ is the octanol-air partition coefficient and $K_{OW}$ is the octanol-water partition coefficient. The second ratio $G_{L2}/G_{12}$ depends primarily on $G_{12}$ the rate at which chemical can diffuse to and from phase 2 (assuming $G_{21} = G_{12}$) which is, to a first approximation, independent of the chemical. If there is no (or negligible) degradation of chemical in phase 2 $G_{L2}$ will depend only on the rate of growth which is also independent of the chemical properties.

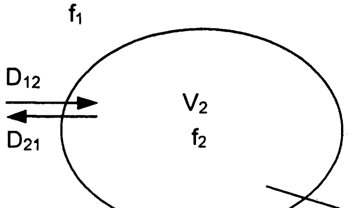

*Figure 3. A plant "phase" of foliage or roots exchanging with the surrounding abiotic phase, either air or pore water.*

For persistent chemicals the ratio $G_{12}/G_{L2}$ is thus independent of the chemical and is conveniently combined with the lipid content, L, and is

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designated A. The ratio of fugacities is then given by $f_2/f_1 = 1 / (1 + K_{OW} / A_W)$ or $1 / (1 + K_{OA} / A_A)$ where the parameters $A_W$ and $A_A$ are characteristic of the growth, diffusion rates, and lipid content. Typical values can be obtained empirically or from theory. When $K_{OW}$ equals $A_W$, there is a 50% approach to equilibrium. At lower $K_{OW}$ values $f_2$ approaches $f_1$ and at higher values $f_2/f_1$ is reduced and kinetic control applies.

We employ this simple correction for non-equilibrium to foliage and roots using $K_{OA}$ and $K_{OW}$ respectively.

In the case of foliage ans stems (collectively referred to as "shoots") $Z_2/Z_1$ is L $K_{OA}$ where $K_{OA}$ is the octanol-air partition coefficient. Ignoring degradation, the fugacity ratio $f_2/f_1$ is controlled by $K_{OA}$ and the relative rates of diffusion to the leaf and growth, i.e., A is now $k_1/(L\ k_G)$. McLachlan *(10)* suggests that $f_2/f_1$ is approximately 1.0 when log $K_{OA}$ is < 8.5 but at higher log $K_{OA}$ $f_2/f_1 <1$ as a result of failure of diffusion to the leaf to overcome growth. Even if growth stops a considerable time is required for equi-fugacity to be acheived and this time may exceed the lifetime of the leaf. An approximate expression for $f_2/f_1$ is then $1/(1+ K_{OA} / 10^{8.5})$

Uptake by roots is mainly by transpiration of soil pore water into the xylem. The partition coefficient is now $K_{OW}$ assuming that the capacity of the root is expressed as an octanol equivalent volume fraction, L. A is then the ratio of the rate of transpiration of water to the rate of growth of lipid. For example water may transpire at 4 mL/h or $cm^3$/h and the root may grow by 100 mL in 100 days or 700 hours, i.e., 0.14 mL/h, of which the lipid is 1%, i.e., it grows at a rate of 0.14 x 0.01 or 0.0014 mL/h. So A is 4/0.0014 or approximately 3000.

For the small mammals such as shrews, and for worms we assume equi-fugacity with the soil recognizing that this may underestimate the extent of biomagnification.

## Model 2. Static Partitioning including Biota

In Model 2 a lower fugacity in the foliage and stem (here referred to as "shoots"), and in the roots is assumed using the above calculations. The selected values of A are $10^3$ for roots and $10^{8.5}$ for shoots. The canopy air, soil invertebrates, and small soil-dwelling animals are assumed to be at equilibrium with the soil.

The results as shown in Table VI demonstrate that the distribution is generally similar to that of Model 1. Order of magnitude estimates of the concentrations in biota are now obtained. Also of interest is the likely fraction of the pesticide mass that is incorporated into the biotic phases.

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**Table VI. The relative amounts (%) of the selected substances in the four phases in soil as calculated by Model 2.**

| | *Pore Air* | *Pore Water* | *Organic Matter* | *Mineral Matter* |
|---|---|---|---|---|
| Atr | < 0.01 | 5.82 | 91.62 | 2.17 |
| Ben | 1.18 | 7.86 | 64.27 | 2.93 |
| Dia | < 0.01 | 1.22 | 98.1 | 0.45 |
| DDT | < 0.01 | < 0.01 | 99.76 | < 0.01 |
| Pen | < 0.01 | 0.016 | 99.86 | < 0.01 |
| Pyr | < 0.01 | 0.016 | 99.87 | < 0.01 |

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## Model 3. Initial Rates of Loss

This model uses the fugacities calculated in Model 2 and loss process information from Table II to estimate rates of loss. In the context of commercial chemicals, when addressing situations of continuing chemical input, for example from product use or losses from municipal or industrial treatment plants, it is often useful to simulate steady-state equilibrium conditions in which there is a constant input rate that is balanced by the sum of the output rates. In this agrochemical context a more appropriate approach is to treat the input as episodic or as a pulse. The model represents an instantaneous picture of the defined mass of chemical in the soil system immediately following application as it partitions, degrades and becomes subject to loss by flows of air, water and solids.

D values must now be defined to express rates of loss from the system. Expressions are suggested in Table II. Environmental transport D values may be calculated by specifying a residence time or characteristic time of the medium, i.e. the time required for it to be completely removed. This is effectively the reciprocal of a loss rate constant. In the case of degradation reactions it is 1.4 times the half-life, i.e. the half-life is ln(2) or 0.693 of the characteristic, or residence, time. The residence time of the air is calculated from a wind speed of 1 m/s and the water residence time represents a leaching rate of 2 mm/day. These rates can be changed to reflect different meteorology or irrigation practices.

In this case, losses from vegetation and fauna are not included, the focus being only on the soil. Since volatilization losses represent sequential losses from soil to air characterised by $D_V$ followed by air advection or outflow characterised by $D_A$, the corresponding resistances are added, i.e. $D_{VO}$, the net volatilization, is the reciprocal of the sum of the two series resistance or reciprocal D values, or $1/D_{VO} = 1/D_V + 1/D_A$.

The total D value for all loss processes can be calculated, and knowing the fugacity the individual and total rates can be estimated as Df. The fastest

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processes become apparent and the rates of off-site loss can be estimated. If equilibrium is maintained during the loss processes, the overall loss is first-order and a half-life can be deduced. The characteristic time for the total loss is $M/f\Sigma D$ and the overall half-life is 0.693 of this time.

Table VII gives results for the selected chemicals and clearly shows the substantial rates of volatilization of the volatile chemicals and leaching of the more water soluble chemicals. In these cases there may be an incentive to undertake more detailed modeling of these key processes. At this stage it is now clear how the chemical is likely to partition and which processes are primarily responsible for transformation and transport from the site of application. The overall half-life is also shown and can be used for a preliminary discrimination between more and less persistent substances.

**Table VII. Chemical loss rates immediately following application of 1000 g of the chemical as calculated by Model 3.**

| | *Degradation mg/h* | *Leaching mg/h* | *Net Volatilization mg/h* | *Total loss from Soil mg/h* | *Residence Time days* |
|---|---|---|---|---|---|
| Atr | 406 | 64.6 | 0.40 | 471 | 88 |
| Ben | 440 | 87.4 | 791 | 1320 | 32 |
| Dia | 407 | 13.5 | 0.021 | 420 | 99 |
| DDT | 7.5 | 0.047 | 0.0025 | 7.6 | 5500 |
| Pen | 481 | 0.17 | 0.008 | 481 | 87 |
| Pyr | 40.7 | 0.18 | 0.055 | 41 | 1020 |

## Model 4. Dynamic Non-Equilibrium Model

The primary weakness of the steady-state equilibrium models are their assumption of equilibrium. This assumption is removed in this fourth model by defining a number of compartments that are believed to have specific and different fugacities. We suggest that six compartments be defined, each having internal equilibrium and thus a single fugacity, but inter-compartment equilibrium does not apply thus the six fugacities differ. The six compartments are

- Soil (pore air and water, organic and mineral matter)
- Root vegetation (primarily in contact with the soil)
- Above ground vegetation (primarily in contact with the air)
- Soil invertebrates (e.g. worms)

- Small soil-dwelling animals (e.g. mice, shrews)
- Atmosphere around the vegetation canopy including aerosols (here referred to as "canopy air")

The expressions used in Models 2 and 3 to account for the lower biotic fugacities no longer apply. This model is illustrated in Figure 4 in which each arrow represents a possible transfer or transformation process. In this case a differential mass balance equation is set up for each compartment and the set of six equations is solved by numerical integration starting with an initial boundary condition representing the quantities of applied pesticide.

Estimates are required for transfer rates or D values to and from the plants and invertebrates and mammals. The following assumptions are made.

For air to foliage exchange it is observed that the rate at which air contacts foliage corresponds typically to 0.5 $m^3$ of air per day per gram (wet weight) or

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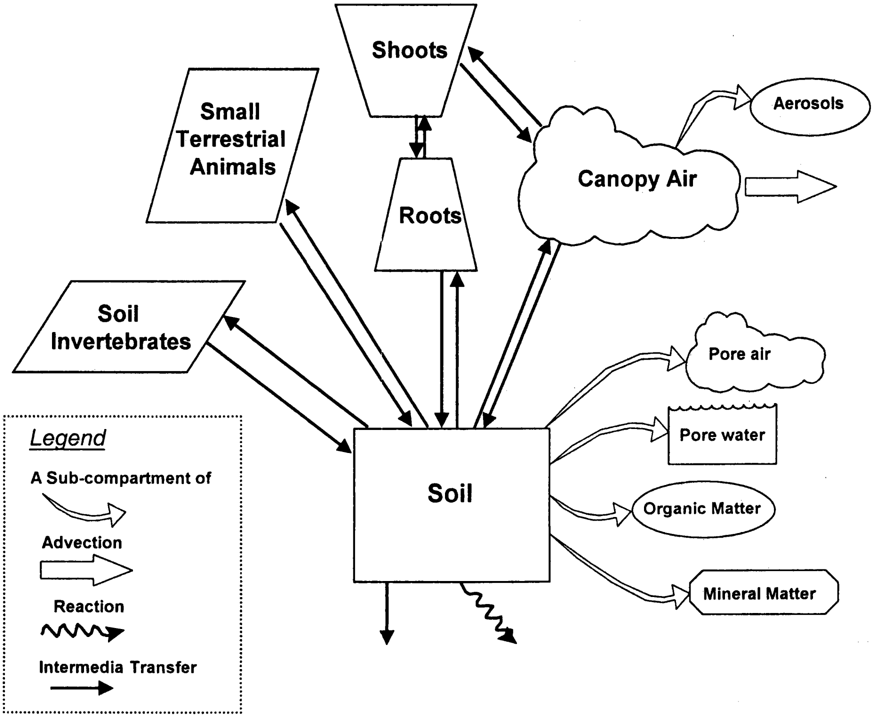
Shoots
Small Terrestrial Animals
Roots
Canopy Air
Aerosols
Soil Invertebrates
Pore air
Pore water
Soil
Organic Matter
Mineral Matter
Legend
A Sub-compartment of
Advection
Reaction
Intermedia Transfer

*Figure 4. The model environment showing the possible transfer and transformation processes in Model 4; the dynamic model.*

$cm^3$ of foliage *(11)*. For simplicity, we treat all above-ground vegetation as foliage. The D value $D_{Shoot}$ is thus of the order of magnitude of $5 \times 10^5/24\ V_{Shoot}Z_A$ where $V_{Shoot}$ is the volume of the foliage, $Z_A$ is the Z value of the chemical in air and $5 \times 10^5$ is the ratio of the air encounter rate (0.5 $m^3$/day) to the unit volume of foliage ($10^{-6}$ $m^3$ or 1 $cm^3$). The characteristic time for uptake and clearance is thus $V_{Shoot}Z_{Shoot}/D_{Shoot}$ or $Z_{Shoot}/(5 \times 10^5/24)Z_A$ or $x_{Shoot}\ K_{OA}\ /\ (5 \times 10^5/24)$ days where $x_{Shoot}$ is the equivalent octanol content of a leaf (typically 1%) and $K_{OA}$ is the octanol-air partition coefficient. For substances of relatively low log $K_{OA}$, i.e., less than 8, equilibrium is achieved within a few days, but for less volatile, highly lipophilic substances uptake is slower.

For soil to root exchange the rate is controlled by the water transpiration rate. If it is assumed that this corresponds to three times the volume of the root per day, the D value is (3/24) $V_{Root}Z_{PoreWater}$ where $V_{Root}$ is the volume of the root. Paterson et al *(12)* reported a transpiration rate of 5.6 $cm^3$ /h or 134 $cm^3$/day through a soybean root of volume 45 $cm^3$. The characteristic time is then $V_{Root}Z_{Root}/D_{EarthRoot}$ or $Z_{Root}/3Z_{PoreWater}$ days. If the octanol equivalent fraction of the root is $x_{Root}$ (typically 1 to 2 %) then this time is $x_{Root}\ K_{OW}\ /3$. Very hydrophobic substances such as DDT are thus absorbed only slowly by the root and most of the sorbtion is to the root surface.

The above and below ground vegetation can exchange chemical through the xylem and phloem. A simple expedient is to assume a D value in both directions equivalent to the root uptake D value.

If the chemical is biotransformed or degraded in the vegetation this loss process can be included if a half-life is available. In some cases photolysis on leaf surfaces may be significant. Growth of vegetation results in dilution of the absorbed chemical. This can be included as a growth dilution D value equal to $V_iZ_ik_{Gi}$ where $k_{Gi}$ is the rate constant for growth.

For soil invertebrates such as insects or worms it is likely that equilibrium is established quite rapidly, i.e., within days *(13)*. The characteristic time for uptake is presumably controlled by the rate at which the organism encounters pore air, pore water, and organic matter. A simple approach is to select a half-time for equilibrium of, say, 2 days for insects and 5 days for worms *(14)* but these can be adjusted in the light of empirical data. If a half-life is $t_{½}$ then the rate constant $k_{EarthWorm}$ is $\ln(2)/t_{½}$ or about $0.693/t_{½}$ $days^{-1}$ and the D value for both uptake and clearance is $V_{Worm}Z_{Worm}k_{EarthWorm}$ where the volume and Z value are those of the invertebrate.

For mammals such as mice or shrews, uptake is likely to be slower and primarily from food but some biomagnification may occur. There may also be metabolic losses. Hendriks et al *(14)* have reported data for biomagnification factors (BMF), from worms to shrews, a BMF of 5 being typical. This can be simulated by assuming that the mammal consumes a multiple of its own mass of food per day. A conservatively high value of this feeding rate multiple is 1.0 (g food/day)/(g body weight) *(15)* . The D value for uptake from consumption of

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worms with a Z value of $Z_{Worm}$ is thus 1.0 $V_{Shrew}Z_{Worm}$ and the D value for clearance is this value divided by the assumed BMF of 5. The net result is that the organism approaches a fugacity about 5 times the worm fugacity. Again, if data are available on metabolic conversion these can be included, effectively as a degradation rate constant within the animal.

The model thus generates estimates of fugacity, concentrations, masses, and fluxes of the substance at selected time intervals during the growing season. Of particular interest are the fractions of the initial mass of pesticide that become subject to various fate processes. A complete accounting of the initial mass is retained, thus at any given time the quantity of pesticide that remains, has degraded, has migrated off-site, or is present in harvestable crops can be estimated.

Figure 5 depicts the time course of fugacity changes in the system for atrazine applied to a 1 ha field at a rate of 1 kg/ha. Initially the fugacity in the soil is high, but it falls steadily as a result of transport from the system, transfer to biota, and transformation. Fugacities and hence concentrations in roots, worms, and mammals are initially low but they rise as equilibrium is approached with the soil. They then fall in concert with the decreasing soil fugacity. The above ground vegetation is less contaminated because it is primarily exposed to the less contaminated atmosphere.

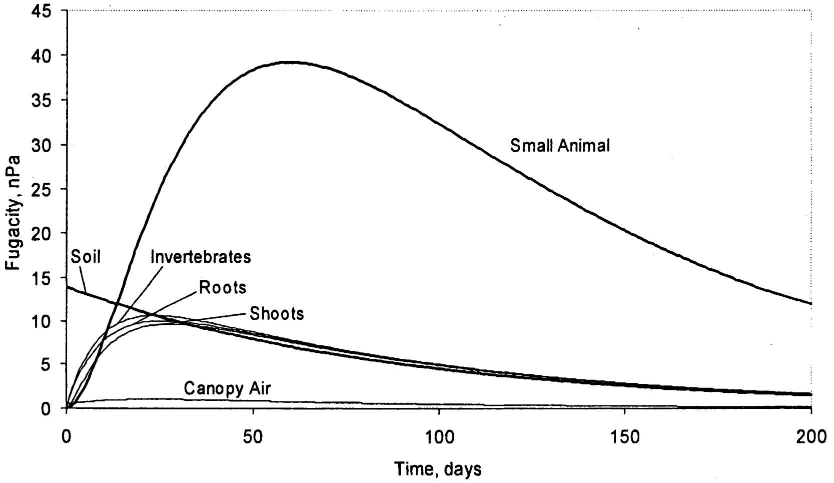

*Figure 5. The time course of fugacity changes for atrazine as calculated by Model 4. The fugacity in the soil declines through-out the 200-day simulation, but there is an initial rise in the fugacities of all other compartments while transfer processes dominate until transformation becomes the dominate process.*

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Monitoring data in the form of periodic concentration measurements can provide confirmation (or otherwise) that these values are reasonable. Even if there is significant disagreement, this information is useful because it indicates that our understanding is flawed. This may be attributable to an erroneous parameter value such as a degradation rate constant or to neglect of an important process. The input parameter or false assumption can then be corrected, thus improving the model and reflecting this new understanding.

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## Discussion

The objective of the simple models described here is to provide a first approximate, quantitative description of the fate of the pesticide. The accuracy is judged to be of the order of a factor of 10 and thus probably inadequate for risk assessment or registration purposes. Even these order of magnitude estimates may be sufficient to demonstrate that a specific process is unimportant and there is little merit in devoting effort to obtaining more accurate parameter values. For example, the evaporation rate may be so fast that degradation kinetics become unimportant. The models thus help to focus attention on the processes that merit closer examination using other models or empirical investigations. A major benefit of a simple model is that its transparency enables the causes of sensitivity or lack of sensitivity to be readily identified by inspection of the equations. Additionally, it can be used to explore how changing pesticide properties are likely to affect fate and persistence.

Determining the relative importance of processes or parameters can be done more formally by a sensitivity analysis in which the input data are systematically varied and the effect on the desired output quantity is assessed. This can be done on each input quantity individually or collectively using a Monte Carlo analysis.

The simple models presented here should thus be viewed as preliminary steps towards justifying the use of more detailed models such as PRZM *(1)* and GLEAMS *(3)*, and organism-specific bioaccumulation models for terrestrial, aquatic, or avian species. The simple and detailed models are thus complementary and not competitive. As was stated earlier, environmental science can best contribute to the more sustainable, effective, and minimal risk use of pesticides by developing a fuller understanding of fate and then of effects. Models ranging from the simple models described here to more complex models taking into account soil transport processes and bioaccumulation phenomena can, we believe, play key roles in this process.

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## Chapter 3

# Pesticide Risk Indicators: Their Role in Minimizing Off-Site Impacts of Pesticides on Water Quality

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Systematic methods that allow a relative assessment of off-site impact of pesticides are of great value to pesticide users, natural resource managers and regulators in choosing the pesticides and practices with the least detrimental impact on the environment. Risk indicators are regarded as useful tools in minimizing off-site impacts of pesticides and can assist in decision-making and policy formulation. While several theoretically attractive models are available, their use is limited due to the lack of availability of input data and local variablity. In contrast, pesticide risk indicators which are not data hungry, are becoming popular, especially to integrate several aspects of concern associated with pesticide use. This chapter provides an overview of available pesticide risk indicators as tools for risk assessment and decision-making for risk management covering both groundwater and surface water. Potential applications of risk indicators are discussed and an example using Pesticide Impact Rating Index (PIRI), is used to demonstrate their application for risk assessment for a specific target group of organisms in a receiving environment.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Pesticide users, natural resource managers, regulators and other environmental and health agencies are increasingly employing risk assessment and decision-making tools in choosing the pesticides and practices with the least detrimental impact on the environment. Such tools are often referred to as "risk indicators" - a term that has been widely used to cover a range of decision support systems, hazard rankings, pesticides risk indices and other tools (*1*). Risk indicators are regarded as very useful tools in minimising impacts of pesticides on non-target organisms and can potentially assist in achieving a range of outcomes, (1,*2*), as described below. The Environmental Impact Quotient (EIQ) of Kovach *et al.* (*3*), the Hornsby Index for water quality (*4*), the GUS Index (*5*) and the AF index (*6*) for groundwater are examples of some of the early risk indicators. Also, in the past, the mass of pesticide applied and frequency of application was seen to be a good risk indicator. However with the advent of more specific, potent and low dose pesticides, there has been a shift in this thinking (1). In recent years, a range of risk indicators have been developed with a variety of objectives in mind. These have been comprehensively reviewed by several workers over the last decade (*2,7,8*).

The available risk indicators differ greatly in terms of their scope, methodology and target user groups (grower, regulator, policy maker etc.). The risk indicators potentially are of great benefit to

- Growers and other pesticides users in choosing relatively environmentally benign pesticides and facilitating compliance with environmental management systems;
- Natural resources managers in identifying risk management options, potential hot-spots and evaluation of management practices;
- Researchers and resource managers in developing appropriate monitoring programs; and
- Regulators and policy makers for analyzing risk trends and developing appropriate policy interventions.

In this chapter we first provide a brief overview of the available risk indicators from Europe, North America and Australasia, discussing their scope and comparability of results. This overview highlights the diversity of compartments covered by these tools and differences in their assessment and therefore the need for a modular approach for risk indicators. Considering the importance of the water quality component of risk assessment for pesticides, we focus on specific water quality risk indicators covering both surface and groundwater quality. Case studies are provided for a water quality risk indicator, namely Pesticide Impact Rating Index (PIRI), that we have developed (*9*).

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## Available Risk Indicators

A wide variety of approaches and tools have been developed to assess the impact of pesticides on the environment. These tools have been reviewed by a number of workers in recent years (*2,7,8,10*) The approaches used vary considerably in complexity and comprehensiveness, include tabular databases, single- and multiple-parameter hazard assessments, composite impact rating systems, a combination of economic and site-specific parameters, and holistic assessments that include agro-ecological impacts and pest-control practices (*2, 11*). The objectives of these approaches may include assessments of the toxicity of pesticides to a particular organism (e.g. honeybees), the potential impact of pesticides on the health of farm workers, the suitability of a pesticide for an Integrated Pest Management (IPM) system or the use of the approach as a decision-making tool for choosing a pesticide with minimum potential for water contamination. Consequently, the approaches differ in terms of the properties of pesticides taken into account and their emphasis on various components of the environment (*2,11*). While Reus and co-workers (*2*) provided an overview of eight European risk indicators that were included in a EU project CAPER (Concerted Action on Pesticide Risk Indicators), the Environmental Risk Program of Cornell University (Lois Levitan) provides a list of a range of available risk indicators with hyperlinks to these individual tools (www.cfe.cornell.edu/risk/PesticideRiskIndList.html).

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Over the last decade, a number of risk indicators have been developed around the world. A list of some of these risk indicators from Europe, Australia and New Zealand is provided in Table I, with a description of the compartments covered and the approaches underpinning the assessment. It is notable from the sample included in the table that a wide variety of compartments and approaches are considered. Two of these indices, namely PRI (*12*) and SafeGauge® (*13*), are specifically designed for a particular industry group (apple and pear growers or sugarcane growers). The PRI is designed to minimize pesticide use on the basis of a range of considerations (including farm workers, beneficial insects and economic consideration, whereas the SafeGauge® is developed to help on-farm management in order to minimize off-site migration of pesticides to surface water only, based on parameters such as pesticide application method, weather conditions and soil types in the farm being assessed. On the other hand the GROWSAFE® (*14*) calculator from New Zealand utilizes the modeling results (based on long-term weather scenarios and probability assessment) associated with specific scenarios with a range of crops to provide guidance to protect groundwater quality only. The EcoRR (*15*) considers toxicity to a range of terrestrial and aquatic organisms from the point of view of assessing the impact of pesticides on biodiversity. PIRI focuses on water quality (surface water and ground water) only and includes toxicity consideration to organisms at different

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**Table I. Compartments considered by a range of pesticide risk indices used in Europe and Australasia. The information on European indicators is taken from Reus et al. (*2*).**

| *Compartment considered* | *EYP* | *HD* | *SYNOP* | *p-EMA* | *Ipest* | *EPRIP* | *SyPEP* | *PERI* | *PRI* | *SafeGauge®* | *GrowSafe®* | *EcoRR* | *PIRI* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groundwater | ✓ | ✓ |  | ✓ | ✓ | ✓ | ✓ |  |  |  | ✓ |  | ✓ |
| Surface Water | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |  |  | ✓ |
| Soil | ✓ | ✓ | ✓ |  |  | ✓ |  | ✓ |  |  |  |  |  |
| Air |  |  |  |  | ✓ | ✓ |  | NS |  |  |  |  |  |
| Human |  |  |  | ✓ | ✓ | ✓ | partially | NS | ✓ |  | partially |  | ✓ |
| Aquatic | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | NS |  |  |  | ✓ | ✓ |
| Earthworm | ✓ | ✓ | ✓ |  |  | ✓ |  | NS |  |  |  | ✓ |  |
| Honeybees |  |  |  | ✓ |  |  |  |  | ✓ |  |  | ✓ |  |
| Source | *2* | *2* | *2* | *2* | *2* | *2* | *2* | *2* | *12* | *13* | *14* | *15* | *9* |

EYP = Environment Yardstick
HD = HD
SYNOP = SYNOP-2
p-EMA = Environmental Performance Indicator of Pesticides
Ipest = Pesticide Environmental Impact Indicator
EPRIP = Environmental Potential Risk Indicator for Pesticides
SyPEP = System for Predicting the Environmental Impact of Pesticides
PERI = Pesticide Environmental Risk Indicator
PRI = Potential for Residue Index
EcoRR = Ecological Relative Risk
PIRI = Pesticide Impact Rating Index, NS – compartment not specifically considered –non-target organisms are considered in general.

trophic levels in aquatic ecosystems or mammalian toxicity of life-time health advisories for drinking water purposes.

Due to major differences in approaches and site-specific applications, it is not possible to make a comparative assessment of all these risk indicators. Depending on the objective the user has in mind, all of these are potentially very useful tools. However, it is important that an indicator is appropriate for the particular purpose and it needs to be carefully chosen, as discussed below.

## Choosing the Right Risk Indicator

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Not all risk indicators are appropriate for all situations. The most important consideration in choosing the risk indicator is the objective of risk assessment and consideration of the question "risk to what and where"? Often the toxicity to target receptor organism has a greater influence on the risk charcaterisation than the environmental fate properties of the pesticide. The available risk indicators often cover a number of compartments and therefore integrate a very wide variety of risk factors. As mentioned earlier, pesticide environmental risk indicators vary greatly in terms of their purpose, compartments and methodology (*2*) and are often very broad in scope covering, for example, the impact on aquatic organisms, soil organisms, beneficial organisms (bees), occupational exposure and human health effects. Furthermore the approach underpinning the assessment varies widely too, from simple scoring and weighting to fuzzy logic and complex modelling. Therefore the results from these indicators are often not comparable. For example, Levitan (*8*), based on a comparison of assessments made by three different methods, demonstrated that the rank order of pesticides depended, in part, upon the components of the analysis. She described the differences between the indices as

*"the pesticides considered, the variables assessed, the choice of specific measurable endpoints as the indicators of impacts on these variables; the mathematical structure of the model, including relative weighting of variables and scoring of results; the method for filling data gaps; and whether usage data are factored into the equation".*

More recently, Reus and co-workers (*2*) compared and evaluated eight pesticide risk indicators that had been developed in Europe and observed a large variation in environmental compartments considered and risk ranking of pesticides.

From an assessment involving 15 pesticides across the eight indicators, they observed that the overall ranking of the pesticide differed when the score of pesticides were assessed for the environment as a whole. Figure 1 demonstrates the variability observed in results from the various risk indicators (*2*). The results were, however, more comparable among the risk indicators when only common compartments (surface water, groundwater and soil) were considered.

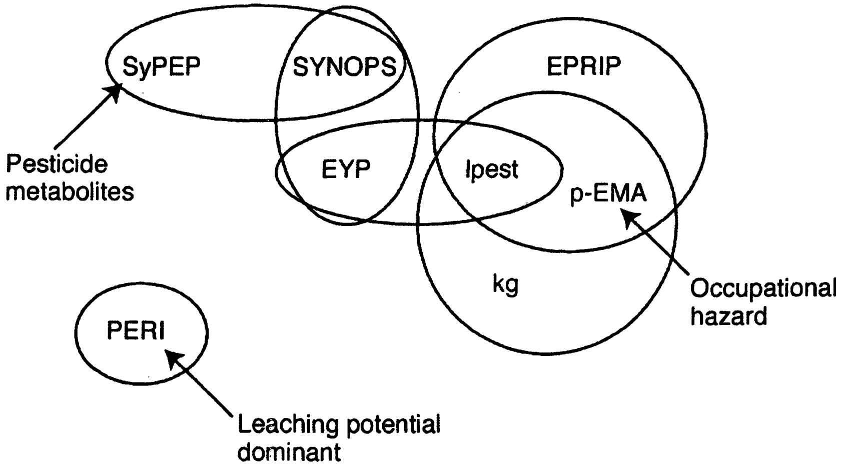

*Figure 1. An ordination of results from seven risk indicators based on 15 pesticides (taken with permission from Reus et al. 2002; copyright Elsevier). For details on the risk indicators abbreviated here, refer to Table 1 and the original source (Reproduced with permission from reference 2. Copyright 2002 Elsevier.)*

Ideally, an indicator needs to deal not just with the inherent hazard of a pesticide but rather with the potential risk it may pose under a given set of conditions of its use. This involves taking into account the rate and method of application of the pesticide as well as environmental and site conditions (*2,9*). Clearly, the choice of the specific tool or risk indicator should be made carefully, considering the environment component of interest (e.g. surface water quality, groundwater quality, beneficial organism), a range of other factors such as:

- the objective of the assessment (i.e. for monitoring decisions, communication tools, shortlisting for second tier assessment);
- the precision required versus the method of risk assessment employed (scoring system versus probabilistic assessment);
- the input data required and their availability (e.g. some risk indicators may utilize locally based data or spatial linkage of georeferenced data);
- the data from previous application of the tool, calibration and validation information; and

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- the ease of use, available skills, assistance and support availability.

In the following section we focus on the risk indicators developed for water quality assessment.

## Risk Indicators for Water Quality

Clearly, the pesticide impact on water quality has financial, social and environmental implications. The key concern is the subtle and chronic effects of pesticides on ecosystem and human health, to which it is difficult to put an equivalent dollar value. However, the worldwide environmental costs of pesticide use have been estimated to be as high as $100 billion per year (*16*). The impact of pesticides on water quality also has important direct financial implications (*17*). Pimental (*18*) reported a total environmental and social cost of pesticide use in the USA to be greater than $8000 million per year and noted a cost of $1800 million per year associated with groundwater contamination due to pesticides alone. Similarly, Pretty and co-workers (*19*), during their assessment of externalities associated with the agriculture in the UK in 1996, noted that out of the total external cost (£2343 million per year), the damage to the natural capital (water component) consitituted about 10% of the total cost (£231 million). In the water component, the exernalities due to pesticides accounted for about half the costs. In particular, the costs associated with pesticides in sources of drinking water were conservatively estimated to be £120 million. Risk indicators targeted at water quality alone have the opportunity to make significant social, economical and environmental contributions.

### Targeted Compartments or Modular Assessment of Pesticide Impact

Some of the earlier work on risk indicators was targeted on specific compartments, such as leaching and contamination potential of pesticides to groundwater: e.g. the AF index of Rao and co-workers (*6*), DRASTIC of Aller *et al.* (*20*) , GUS index of Gustafson (*5*). The AF index has been widely used in the USA in the GIS framework for modelling non-point source pollution of groundwater by pesticides (*21*). Rather than a scoring system, in some risk indicators (e.g DRASTIC) the AF index was mechanistically based. Modification and improvements in the AF index have been made since by Lee *et al.* (*22*) to represent the uncertainty in pesticide leaching assessment and by Kookana and co-workers (*9*) to include variation of organic carbon content and soil microbial population density with depth in the soil profile.

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In New Zealand, to address potential groundwater contamination by pesticides, a decision-support tool, the GROWSAFE® Calculator, was developed by Hort Research New Zealand (www.hortresearch.co.nz), based on industry-typical spray diaries for 34 of the major crops grown in New Zealand. The principal aim of the product was to provide advice as to which chemicals can be selected for local conditions to minimise the likelihood of leaching, and the risk of soil build-up of pesticide residues (*14*). The Calculator utilizes the results of simulations done by the SPASMO-Pesticide (Soil Plant Atmosphere System Model) model using input data supplied by grower bodies, the pesticide manufacturers, and Regional Councils, and then interrogating it for a particular case. SPASMO is a mechanistic model that predicts the transport of water through the rootzone, and simulates the fate of chemicals (*23*). The Calculator covers some 850 combinations of crops, regional climates, and soil types across New Zealand.

SafeGauge® was designed for better management to minimize off-site transport of pesticides. It utilizes geo-referenced soil properties and weather data for sugarcane production regions in Queensland, Australia and can therefore make useful predictions based on long-term weather data. SafeGauge® was designed for the Queensland sugarcane industry because there was an extensive inventory of pesticide use data available to underpin it. While SafeGauge® predicts potential off-site movement of pesticides in relation to management practices, it does not take any pesticide toxicity into account.

Specifically, to cover only the surface and groundwater water quality compartments, we have developed a risk indicator, in the form of a software package, named PIRI (Pesticide Impact Rating Index). It provides an improved pesticide risk indicator for water quality. PIRI can be used to make relative assessment of pesticides (*9*) as well as a spatial (GIS based) assessment of a pesticide under different land uses (*24*). In the following section we describe PIRI in more detail and show its application through a case study.

## Pesticide Impact Rating Index (PIRI)

In contrast to some other risk indicators covering a range of compartments, PIRI focuses solely on assessing off-site migration potential of pesticides and risk of contamination of surface water or groundwater. PIRI does this with a more rigourous mathematical treatment and yet maintains lower requirement for input data. Furthermore, it allows consideration of potential toxic effects on aquatic organisms and a comparison with drinking or environmental water quality guidelines. This risk indicator considers site specific pesticide use, the pathway through which the pesticides are expected to migrate to the water resource (asset), and the value of the asset being threatened. Each component is quantified using site conditions (e.g. soil type, soil organic matter content, water input, slope of land, soil loss, recharge rate, depth of water table) and environmental conditions (e.g., rainfall and temperature). For each pesticide, the rate and method of application, its sorption and persistence properties and its toxicity to a range of receptor organisms (chosen to represent different trophic levels, e.g., algae, *Daphnia*, fish and rat) are assessed. A detailed description of the mathematical basis for integrating various risk factors in PIRI has already been published by Kookana *et al.* (*9*).

## Example Output of PIRI

Although PIRI results are calculated quantitatively using a mathematical approach, the results are then classified into categories such as low, medium or high risk. Assessment of both potential off-site migration (mobility) as well as risk to specific target organisms (e.g. fish, *Daphnia*, algae) is made by combining mobility and toxicity considerations. Figure 2 shows the example output in terms of potential mobility as well as mobility+toxicity of pesticides.

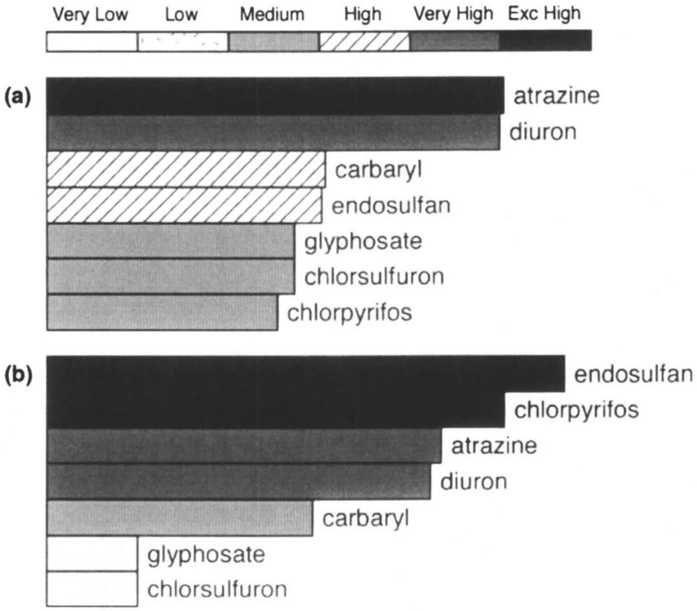

*Figure 2. An example of PIRI output showing risk rating for potential (a) mobility and (b) mobility + toxicity of pesticides to fish.*

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As can be seen from the figure, while atrazine and diuron had much greater potential of off-site migration (Figure 2a) than endosulfan and glyphosate, when toxicity of the pesticides to fish was considered, endosulfan was rated the highest risk compound, glyphosate the lowest risk and atrazine and diuron were downgraded in risk due to their lower toxicity to fish (Figure 2b).

The example presented here highlights the fact that the risk profile of pesticides is dependent on the question of what is being protected. Clearly the choice of specific receptor has a strong bearing on the risk from pesticides. Therefore, the inherent risk rating of pesticides without due consideration to the toxicity to the receptor organism is not very useful. For a holistic assessment, there is a need to include several receptor organisms representaing different trophic levels (e.g. fish, *Daphnia*, algae, mammals) and to profile them in a manner shown in Figure 3.

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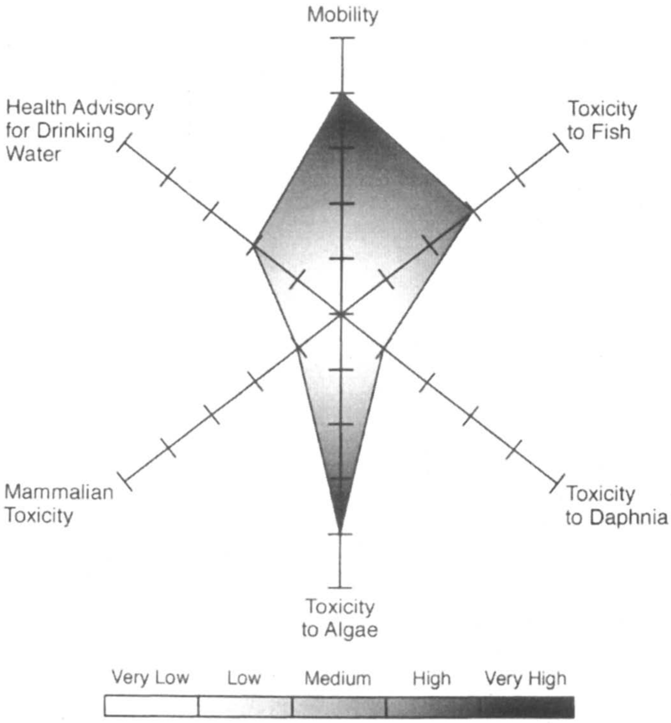

*Figure 3. An overall evaluation map describing the variable risk rating of a pesticide for a range of organisms (example only).*

# A Case Study Using PIRI Risk Indicator

## Potential Risk from Pesticides used in Banana Plantations on Shrimp and Crustaceans

Banana production, a main source of export income for Ecuador (37%) and Costa Rica (20%), relies on a range of pesticides. Off-site migration of pesticides from banana plantations and their potential impact on shrimp farms located along the Guayas River basin of Ecuador has been a matter of concern. Therefore, an assessment of off-site migration potential of pesticides was made using PIRI. A survey of banana farmers in the Guayas River Basin Area Ecuador (Caceres, T; Atomic Energy Commission) and La Suerte River Basin in the Atlantic lowlands of Limon, Costa Rica (*27*), indicated that some of the commonly used pesticides are propiconazole, chlorothalonil, imazalil (fungicides), cadusafos, terbufos, chlorpyrifos (organophosphates), carbofuran (carbamate), and ametryn (triazine herbicide).

The frequency of use for fungicides is reported to be very high. For example, repeated aerial sprayings per year of fungicides is a common practice (*25,26,28*). It has been reported that fungicides are applied up to 50 times a year by aircrafts, and nematicide-insecticides, 2-3 times a year. About one third of the imported volume of pesticides into Costa Rica is used in banana plantations, covering about 10% of agricultural land (*25*). However, the actual data on the amount applied and frequency of use for each of the above pesticides are not readily available and may vary from season to season.

There is a general lack of published data on pesticide toxicity to shrimps. We could only find 26 pesticides for which toxicity data for shrimps were readily available in the public domain. These, however, were not for pesticides used in banana production. In our attempts to find a suitable indicator organism as a surrogate for shrimps, we found that the toxicity data for *Daphnia* and shrimps was well correlated, as shown in Figure 4. *Daphnia* toxicity data was readily available for pesticides used in banana production in Ecuador. Based on this assessment, PIRI indicated that generally cadusafos, carbofuran, propiconazole and terbufos had a high risk of off-site movement (Table II).

While no monitoring data on pesticide residues are available for Ecuador, three monitoring studies downstream of banana plantations were available from Costa Rica to corroborate predictions from PIRI (Table II). Castillo and co-workers (*25,26,27*) carried out monitoring studies and effects of pesticide in surface waters receiving runoff from banana plantations in the Rio Suerte Basin of Costa Rica. The monitoring data only weakly correspond to the rankings given by PIRI. However, certain pesticides such as terbufos, cadusafos and carbofuran which were rated to have high potential for mobility and very high risk to crustaceans (*Daphnia*) were also identified as the pesticides that were

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**Table II. A risk assessment scenario for banana pesticides for mobility and shrimp toxicity (based on data from banana plantations in Costa Rica). Monitoring and application rate data from Castillo and coworkers (*25,26,27*).**

| *Pesticide* | *Application rate in (kg a.i./ha)#* | *Frequency of use over a season (assumed)** | *Rating for potential mobility* | *Rating for potential toxicity to Daphnia* | *Detection frequencies reported (25,26,27)* |
|---|---|---|---|---|---|
| Ametryn | 1.2 | 1 | High | Very Low | 19% (*27*) |
| Cadusafos | 3.7 | 1 | Very High | Very High** | 87% (*27*)<br>Residue levels close to acute toxicity level |
| Carbofuran | 6.0 | 1 | Very High | Very High** | Detections reported after application (*25,26,27*) |
| Chlorothalonil | 1.08 | 5 | High | Very High | 2% (*27*) |
| Chlorpyrifos | 0.5 | 1 | Medium | Very High | 11% (*27*) |
| Propiconazole | 0.4 | 5 | High | Medium | 64% (*27*)<br>Constant presence (*26*) |
| Terbufos | 4.2-5.6 | 1 | High | Very High** | 28%<br>Residue levels close to acute toxicity level |

# Application rates were taken from either Castillo *et al.* (*27*) or from pesticide label.

* Fungicides are reported to applied very frequently (up to 50 application in a season) by Castillo *et al.* (*27*)

** These compouds were identified by Castillo *et al.* (*25*) through multivariate analysis as contributors to shifts in macro invertebrate communities.

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found to be associated with significant community changes by Castillo *et al.* (*25*).

By implication, using the relationship shown in Figure 4, these pesticides would have a high impact on other crustaceans also.

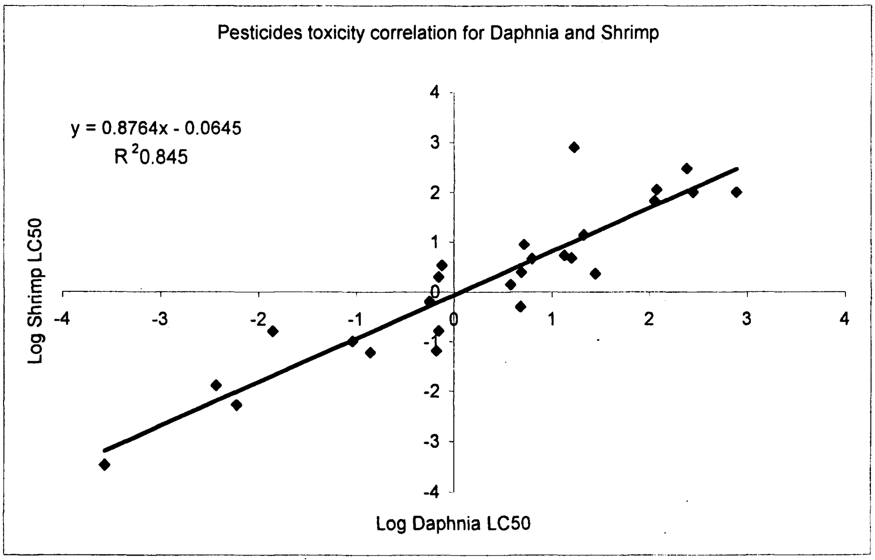

*Figure 4. The correlation between toxicity data (log $LC_{50}$) for Daphnia and shrimps showing that Daphnia data could be a useful toxicity indicator for shrimps*

## Potential Utility of Risk Indicators in Better Management of Pesticides

Risk indicators can be particularly beneficial in:

- Identifying pesticides that pose an unacceptably high risk to environment and/or to identify priority pesticides for targeted monitoring and management,
- Providing a relative assessment between landuses, agricultural practices or agricultural cropping options (e.g. herbicide tolerant crops versus conventional systems),
- Integrating risk factors in order to identify scenarios (based on pesticide

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properties, site conditions and receiving environment) and potential hot-spots needing priority intervention,

- Assisting pesticide users in risk-based decision-making on timing or method of application of pesticides,
- Quantifying the effectiveness of risk reduction measures,
- Describing risk trends over time and assessing regulatory compliance and environmental performance,
- Advising policy makers on appropriate policy interventions,
- Enhancing risk communication, awareness and education, and
- Facilitating "Eco-labelling" or "Green-labelling" and internal auditing to facilitate adoption of environmental management systems.

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Clearly, pesticide risk indicators can be very useful tools for first-tier risk assessment and screening a large number of pesticides from the standpoint of a particular group of organisms. Given (i) the high number of pesticides in use, (ii) competing priorities on resources, and (iii) the limited availability of resources, capacity, and skill in developing countries, it is highly desirable to carry out first-tier assessment for a more targeted follow-up or intervention.

## Acknowledgment

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## Chapter 4

# Development of a Decision-Making Tool to Minimize Environmental and Public Health Risk of Pesticide Application

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Pesticides are widely used in agriculture; however, there are concerns over the effect of pesticides on public health and the environment particularly when they move beyond a field boundary. Risks associated with pesticide use can be minimised if correct management decisions are made. Spray models can be used to assess the deposition of pesticide onto crop and other infield surfaces and to predict the amount of spray drift downwind from the sprayed area. By combining pesticide exposure models, dose response models and decision theoretical tools, various management options can be evaluated to maximise the effectiveness of plant protection products and minimise risks to public health and the environment from agricultural spraying activities.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

## Introduction

Pesticides are widely used in agriculture to control pests (weeds, insects or pathogens) and thereby increase yield and farm income. They remain an essential tool for agricultural industries in the production of high quality produce and are a key component of integrated crop management (ICM) in cropping systems worldwide with some 2.56 million tonnes used per year (*1*). Pesticides are generally applied as sprays to produce coverage of droplets containing the active ingredient on the target (e.g. an insect, leaf surfaces or part of a plant).

Spray may be lost to non-target areas within a crop such as deposition on the soil or non-target plant surfaces and the action of wind may result in spray moving from the spray area. There are increasing concerns over the effect of pesticides in the environment particularly when they move beyond a field boundary. By utilising techniques that will maximise deposition on the spray target, it is possible to both improve the efficacy of pesticide applications and limit the movement of liquid droplets away from their point of release, both within and outside a target area.

For optimum control of pests and weeds in agricultural cropping situations, the grower is required to take careful consideration of many factors. These include chemical selection, crop type, pesticide resistance, crop yield, costs of production, farmers revenue, spraying equipment (e.g. type of sprayer, nozzle selection, operating parameters), spraying techniques (e.g. buffer zones, no spray areas), meteorology (temperature, relative humidity, wind speed and direction), sensitive areas downwind (e.g. non-target crops, livestock, aquatic organisms and areas that people occupy). Managing these factors in an integrated, holistic manner is often very complex. It requires combining tools, resources and information from several sources to optimise the application. Many parameters can also change during application (e.g. wind speed and direction) and application techniques must then be modified to prevent possible contamination of non-target areas. Failure to rapidly and appropriately manage these complex inter-related parameters has been the reason behind many pesticide drift incidents.

Risks to public health and the environment associated with pesticide use can be minimised if correct management decisions are made. For a spray operation to be effective it needs to control pests (and hence increase crop yield and gross income) with a minimum of off-target environment and public health damage. By combining spray models which give pesticide exposure and dose-response models with decision theoretical tools, various management options can be evaluated to maximise the effectiveness of plant protection products and minimise risks to public health and the environment from agricultural spraying activities.

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## Risk Management

Risk assessment is a process that enables management and communication tools to be developed to aid controlling any adverse effects of pesticide applications (*2*). It comprises the discrete steps of identification of source and hazard, dose response, exposure and calculation of risk (*3*). There are acceptable risk management concepts that apply to public health and the environment arising from exposure to pesticides when applied under controlled conditions as pure or mixed formulations. There are two at risk groups to consider for public health (*4*), namely: (i) applicators and other workers (e.g. farm workers); and (ii) the local community (e.g. residential areas, schools and other areas occupied by humans).

In the risk assessment process, exposure studies of biota need to take into account formulation of the pesticide and its bioavailability. It is necessary to combine good quality application data with pesticide chronic toxicity data, generally the data required for registration of a pesticide. The risks to the environment need to take into account affects on: (i) terrestrial species; and (ii) aquatic species (*5*). Other key areas include drinking water area exposure assessments and protection of non-target sensitive crops.

The currently accepted procedures in Australia of the Department of Health and Aged Care (*6*) and the USEPA (*7*) enable the formalized approach of risk assessment to be applied when required. Calculation of dose enables recommendations to be made regarding safe criteria for public health and the environment. The understanding of risk assessment and implementation and management are two sequential steps where assessment is first undertaken followed by development of the management tool based on identified risks. In many cases a complete risk assessment may not be undertaken for practical reasons. For this reason decision making tools are developed to provide a risk-based approach that acts as a framework.

## Modeling Pesticide Exposure

Considerable research has been focused on understanding the movement of sprays from the release point and various computational models have been developed to simulate the spray application process. These spray models can be used to assess the deposition of pesticide onto crop and other infield surfaces (e.g. ground, weeds) and to predict the amount of spray drift downwind from the sprayed area. This can be used to give a measure of pesticide exposure. Runoff is another important exposure route/ pathway for pesticides to affect groundwater or other areas.

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## In-crop

Close to the spray nozzle, droplet numbers are high and the density of the spray can influence the local air turbulence (*8*). The fact that droplets are being propelled from the nozzle in a certain direction causes surrounding air to be entrained into the spray plume (*9*). The combination of the high droplet concentration, initial spray sheet and entrained air can provide a blockage to cross flowing air resulting in regions of low and high air pressure leading to the creation of spray induced vortices (*10, 11*). The spray vehicle (e.g. tractor or aircraft) and spray structures (e.g. booms and shields) can also create additional turbulence in the region where the spray is being produced. These factors can influence the amount of spray drift resulting from the application

Models of droplet movement in the near nozzle region are often ballistic or particle trajectory models and are based around applying equations of fluid mechanics. The two main forces acting on droplets during a typical spraying situation are gravity and drag (*12*). The trajectory of each fluid element is divided into a large number of small discrete time steps of constant duration, during which the velocity components (u,v,w) of the particle are kept constant and each droplet is followed as it moves through the atmosphere. A meaningful estimate of dispersal statistics can be obtained by following a large number of trajectories (*13*).

Vegetation type and structure can play an important role in determining the amount of pesticide exposure. The movement of spray droplets is influenced by vegetative structures within the sprayed area as well as vegetation downwind of the sprayed area. Above ground, a plant's architecture is determined by the size, shape and variety of their individual components and how these components are connected to each other (*14*). Examples of plant components include leaves, internodes, buds, flowers and fruit. Plant architecture is constructed by the repeated production of a relatively small number of components. By treating plant geometry as an arrangement of discrete components in space it is possible to keep model specifications concise, even if the simulations eventually yield extensive structures that are made up of many components (*15*).

Figure 1 shows a simulation of spray deposition on a cotton plant that has been developed using the Lindenmayer systems (L-systems) formalism (*16-18*). By combining spray trajectory and plant architectural models the pesticide exposure on different regions in the crop (e.g. leaves, fruit and ground) can be determined as well as the proportion of spray available to move off the field as spray drift. Irrigation or rainfall events can result in the movement of pesticide that deposit on the ground into nearby streams or ground water.

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## Spray Drift Away From Treated Area

Once a droplet moves away from the spray nozzle it will normally move under the influence of the prevailing meteorological conditions unless it has an (short range) electrostatic charge or other special energy characteristics. At this stage the spray concentration in the air is low, so the influence of the droplets on the local air turbulence is negligible (*8*). Two main approaches have been utilized to determine the amount of spray drift moving away from treatment areas, namely the use of models based upon Gaussian diffusion theory (*19, 20*) and Lagrangian solutions such as AgDRIFT and AGDISP(*21, 22*). Recent work is looking into adding a CALPUFF-type handoff for longer-range dispersion model, e.g. through the model SPRAYTRANS currently under development by the USDA Forest Service (*23*).

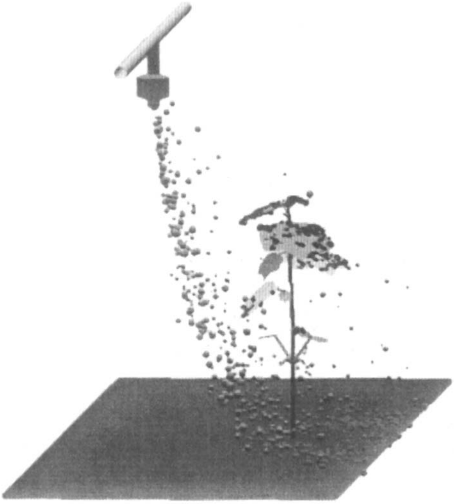

*Figure 1. Simulation of the movement of spray droplets and deposition on a cotton plant (See page 1 of color inserts.)*

Over the last decade, significant research has been conducted to establish and validate spray drift profiles and exposure models (*24, 25*). Models such as AgDRIFT and AGDISP allow the end-user to determine spray drift exposure levels based on various inputs covering all of the major factors involved in spray formation, transport and deposition from aerial and ground-based application systems. Toolboxes allow an assessment of the appropriate protective measures such as no spray buffer zones based on product toxicity information as a fraction of the application rate. AgDRIFT is presented as a three-tiered model allowing screening level and refined assessments depending on labeling needs. AgDRIFT also includes curve fits for deposition from the Spray Drift Task Force orchard airblast and ground application studies, as well as aerial dispersion code validated from extensive field studies (*26*).

Drift management often provides a challenge in spray applications. For example, wind speed and direction conditions can change rapidly during applications, shifting the direction and magnitude of the potential off-target movement of the spray. There are also often conflicting requirements when developing optimum spray application programs. For example smaller droplets may be better for providing greater coverage on the target, yet these small droplets also can pose a greater off-target drift risk. In many cases, it is desirable to apply a narrow droplet size spectrum with as few driftable droplets (often with diameter below 100-200μm) relative to the average droplet size.

## Development of a Decision Tool

A decision theoretic analysis tool is being developed to assist farmers and regulators in evaluating the risks and benefits associated with the use of agrochemicals. It has been developed for attachment to the back-end of the physical models to assist farmers and regulators in evaluating the risks and benefits associated with the use of agrochemicals. For this paper we adopted a simple Excel module, because its usefulness depends in part on accessibility and availability. The analysis takes into account the many factors growers need to consider in making a spray decision.

The purpose of this analysis is to assess, according to established criteria such as the maximisation of expected benefits or minimization of expected risks, the expected consequence for rare or other events (by summation of the product of probabilities and magnitude). The optimal strategy to minimise the expected value of the net benefits, suitably discounted, is to be adopted. This approach can be used to reflect the environmental and public health risks of pesticide application.

### Spray Strategy

A range of control methods (e.g. chemical control, cultural control and biological control) are available to reduce pest numbers in cropping situations. For this analysis we are assuming that the grower has decided to adopt chemical control for a particular crop and pest. Growers may monitor insect population levels and only spray when the pest population is sufficiently high to justify the cost of treatment (*27*) or they may spray without any actual knowledge of pest levels. The later case is often referred to as calendar spraying and is used for

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example for the preventative applications of fungicides, in cases where detection of the pest is too late to prevent significant damage or where there are information gaps (*27*).

Monitoring of a crop is generally achieved by sampling a small section of the crop according some predefined criteria (*28*). While monitoring can give a good indication of pest levels within the crop the results are not always a true indication of total pest level in a crop. For example, no pest may be found on the sampled plants, which would indicate low pest levels when pest levels may in fact be high. No pest may be found because the randomly selected plants had no pests whereas pest are present on most plants, the crops may have been sampled at the wrong time or an inexperienced operator may have looked in the wrong place. Clearly sampling errors can occur in commercial production cropping systems when CV values are high. Consequently we suggest that the variables are random rather than deterministic.

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## Pest Levels

The amount of damage caused to a crop by pests is dependent on the numbers of pest present in the crop. The level of pest infestation at which the reduction in revenue from the crop, due to pest injury, equals the cost of controlling the pest is often referred to as the 'Economic Injury Level'(*28*). The Economic Injury Level can be useful in obtaining an approximate evaluation of the influence of different variables on the 'break-even' level of attack for particular control actions. However, for practical decision making an 'Action Threshold' (AT) (*29*) or 'Critical Density'(*28*) needs to be determined. The Action Threshold may include factors such as;

- Economic Injury Level.
- Empirical 'trial and error' experience.
- Dynamics of the pest population. For example with endogenous pests that build up over time questions concerning the optimal level and timing of control may need to be considered rather than a simple 'break-even' threshold.
- Risk attitude of the grower. This will influence the 'margin for error' that will need to be included to make the action threshold acceptable.

Once an action threshold has been determined for a particular crop and pest, growers will typically monitor the crop and spray only when pest levels are found to be above the action threshold. Action thresholds for many agricultural pests and crops are available through agricultural departments or industry bodies.

## Off-Target Damage

If all pesticide label directions are followed during application of the pesticide, the risk to public health or off-target damage from spray drift is designed to be below the level of concern deemed by regulatory authorities as acceptable. However all spray operations have the potential to generate some off target movement of the pesticide (spray drift) and growers can be legally responsible for pesticide trespassing onto a neighboring property or any off target damage resulting from the application. It is this probability of off-target damage that is usually of greatest concern to the pesticide applicator. Off-target damage to neighboring crops, vegetation, wildlife or aquatic organisms may be observed shortly after application and can at times be very visible (particularly when herbicides are used).

To model the off-target damage from pesticide applications it is necessary to use an appropriate pesticide exposure model (discussed in earlier section) and dose-response model. Probabilistic dose-response models should be appropriate to the hazard being studied and preferably use descriptions of the fundamental biological basis for the hazard (*30*).

In managing off-target damage it is necessary for growers to identify any potentially sensitive areas in the awareness zone around the field to be sprayed (*31*). If any such areas are identified, then spraying should only be undertaken with the wind direction away from the sensitive area or drift reducing techniques (*31*) are used to keep drift below acceptable levels.

## Decision Trees

Decision trees have been used to evaluate spray decisions (*29, 32*) but to date they have generally not been extended to cover environmental and public health risk from the use of pesticides. Figure 2 shows an example of a decision tree for spray management that includes pest levels efficacy and the risk from off-target damage. The decision tree was developed in Microsoft Excel using Lumenaut Decision add-in (*33*).

Income and operating expenses used in the decision tree were based on the average figure for the 2005 cotton crop in Australia (*34*). A summary of the main input values to the decision tree are shown in Table I. Probabilities and values used in this paper are for illustrative purposes only and need to be assessed for each individual case. Information such as yield loss expected due to pest

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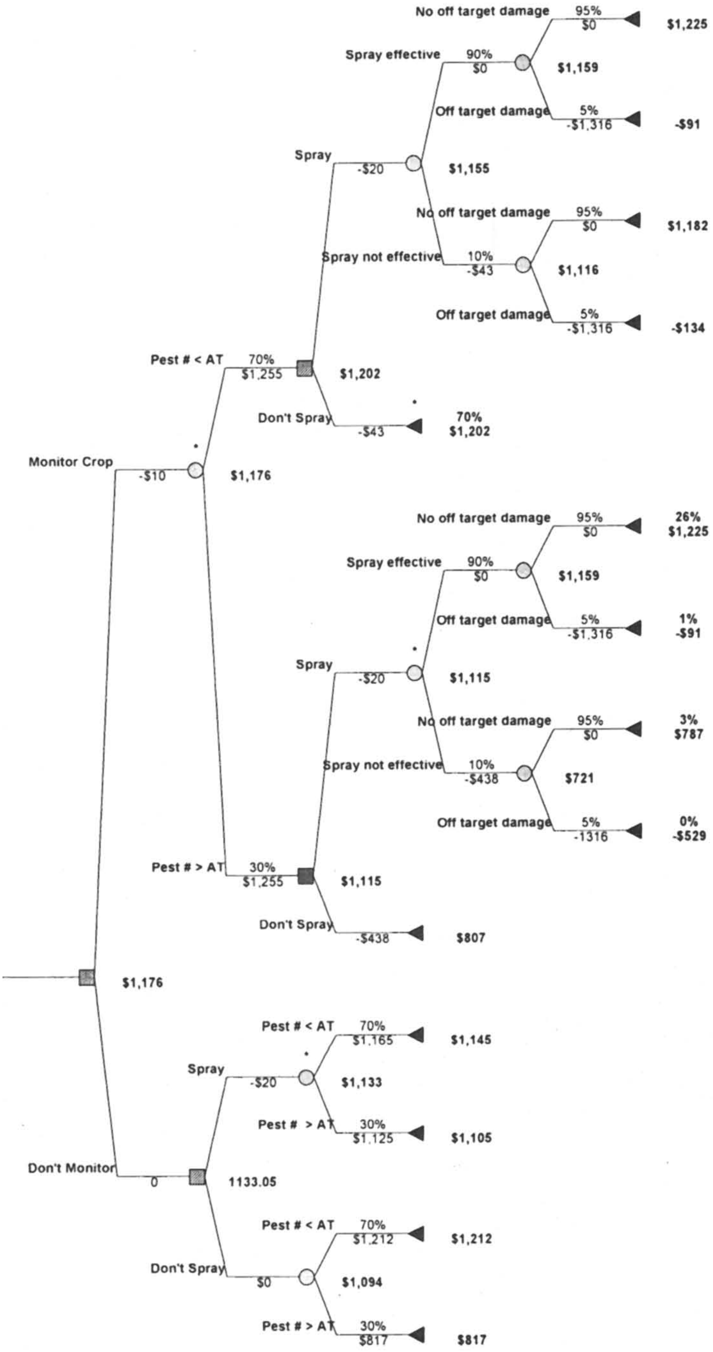
No off target damage
95%
$0
$1,225
Spray effective
90%
$0
$1,159
Off target damage
5%
-$1,316
-$91
Spray
-$20
$1,155
No off target damage
95%
$0
$1,182
Spray not effective
10%
-$43
$1,116
Off target damage
5%
-$1,316
-$134
Pest # < AT
70%
$1,255
$1,202
Don't Spray
-$43
70%
$1,202
Monitor Crop
-$10
$1,176
No off target damage
95%
$0
26%
$1,225
Spray effective
90%
$0
$1,159
Off target damage
5%
-$1,316
1%
-$91
Spray
-$20
$1,115
No off target damage
95%
$0
3%
$787
Spray not effective
10%
-$438
$721
Off target damage
5%
-1316
0%
-$529
Pest # > AT
30%
$1,255
$1,115
Don't Spray
-$438
$807
$1,176
Pest # < AT
70%
$1,165
$1,145
Spray
-$20
$1,133
Pest # > AT
30%
$1,125
$1,105
Don't Monitor
0
1133.05
Pest # < AT
70%
$1,212
$1,212
Don't Spray
$0
$1,094
Pest # > AT
30%
$817
$817

*Figure 2. Example of a spray decision tree*

**Table I. Main input values used in decision tree (Australian Dollars)**

| | | |
|---|---|---|
| Gross income | $4,387/ha | |
| Operating expenses | $3,162/ha | |
| Operating profit | $1,225/ha | |
| Monitoring cost | $10/ha | |
| Application cost | $10/ha | |
| Chemical cost | $10/ha | |
| Income reduction if pest numbers not controlled | | |
| Pest numbers < Action Threshold | $43/ha | (1% of gross income) |
| Pest numbers > Action Threshold | $438/ha | (10% of gross income) |
| Cost of off target damage | $1,316/ha | (30% of gross income) |

numbers should be obtained where possible from reliable independent sources and/or models.

Figure 3 shows the optimal path analysis for the decision tree in Figure 2. This analysis indicates that the optimal strategy is to monitor the crop and base spray decisions on actual pest levels. If pest levels are below the action threshold (Pest#<AT) the optimal strategy is not to spray but if pest levels are above the action threshold (Pest#>AT) the optimal strategy is to spray.

The scenario in Figures 2 and 3 is based on spraying in good conditions with low drift techniques (air induction nozzles) so that there is a high probability (95%) of no off-target damage occurring. If however results from pesticide exposure and dose–response curves indicate that the probability of no off-target damage reduces from 95% down to 50% (all other factors remaining constant) then the optimal strategy is to do nothing (Figure 4).

## Conclusion

Pesticides are widely used in agriculture; however there are concerns over the effect of pesticides on public health and the environment particularly when they move beyond a field boundary. Spray models such as those described in this paper can be used to estimate pesticide exposure. By combining spray models, dose response models and decision theoretical tools, various management options can be evaluated to maximise the effectiveness of plant protection products and minimise risks to public health and the environment from agricultural spraying activities.

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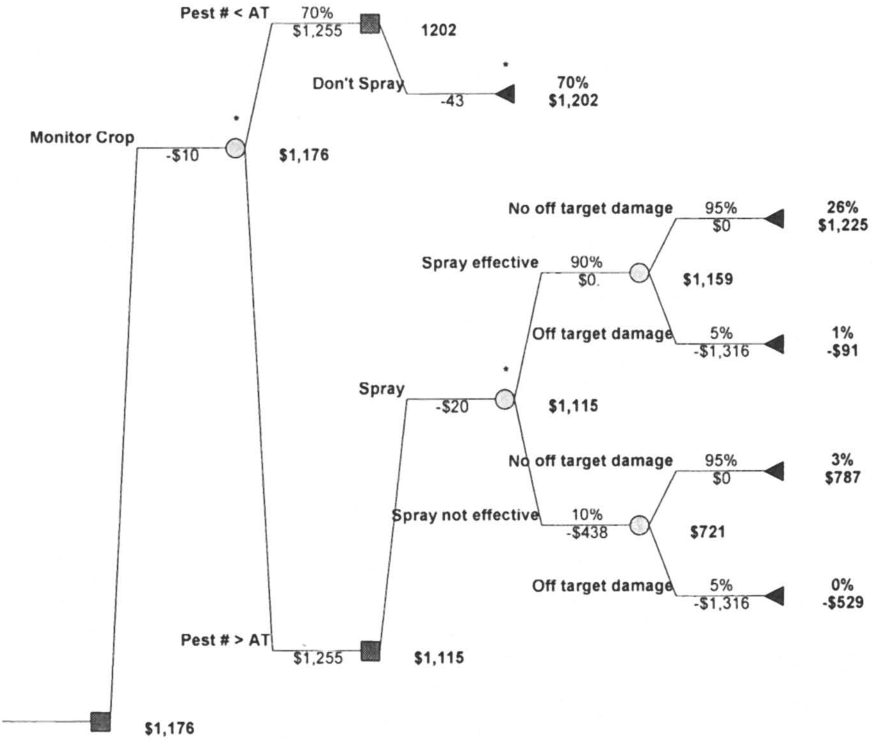
Pest # < AT
70%
$1,255
1202
Don't Spray
-43
70%
$1,202
Monitor Crop
-$10
$1,176
No off target damage
95%
$0
26%
$1,225
Spray effective
90%
$0.
$1,159
Off target damage
5%
-$1,316
1%
-$91
Spray
-$20
$1,115
No off target damage
95%
$0
3%
$787
Spray not effective
10%
-$438
$721
Off target damage
5%
-$1,316
0%
-$529
Pest # > AT
$1,255
$1,115
$1,176

*Figure 3. Optimal path analysis for decision tree in Figure 2.*

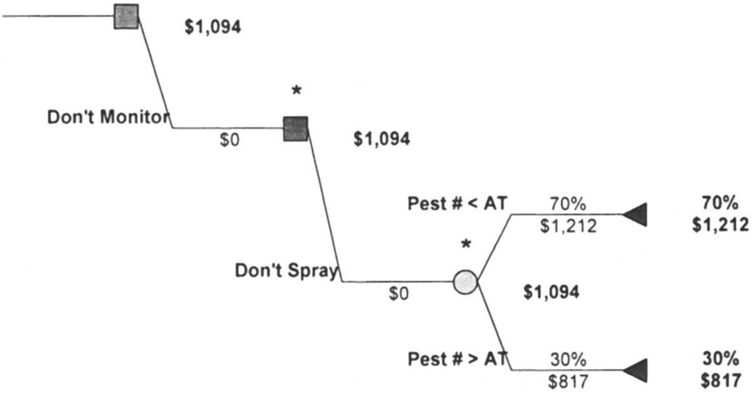
$1,094
Don't Monitor
$0
$1,094
Pest # < AT
70%
$1,212
70%
$1,212
Don't Spray
$0
$1,094
Pest # > AT
30%
$817
30%
$817

*Figure 4. Optimal path analysis for decision tree with probability of no-off target damage reduced from 95% down to 50% in Figure 2.*

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## Chapter 5

# Evaluation of the Standard Quotient and EcoRR Methodologies Based on Field Monitoring from Rice Fields

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Selection of agrochemicals that may control pest outbreaks while ensuring smaller impacts on the environment is important for the success of IPM. Currently, regulators evaluate risks from chemicals following a tiered system which relies heavily on the quotient method. To test the validity of two current models used for risk assessment, impacts of two insecticides and a pollutant on a mesocosms agro-ecosystem were monitored during two rice growing seasons. Whilst some of the observed effects on populations agreed with the models' predictions, inadequacies of such methods stemmed from unknown exposure mechanisms under natural environments, inappropriate toxicity data derived under laboratory conditions and omission of the physical and chemical properties of the compounds in the quotient model. This validation study provides an opportunity to reconsider current risk approaches.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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In recent years there has been a proliferation of methods to determine risks from chemicals released into the environment. Apart from human health risks, confined mainly to workers exposure, most methods are designed to deal with registration of agro-chemicals and pharmaceuticals (*1*) or with ecological risks to non-target species and ecosystems (*2*). The latter methods are not only useful to protect the environment at large but can also be regarded as screening procedures for agro-chemicals that may be used in integrated pest management (IPM) programs (*3*), since most predators utilised in biological control are obviously non-target species for the diverse array of pesticides used in modern agriculture.

Current registration procedures in developed countries follow a tiered system whereby chemicals are evaluated in successive steps from simple hazard assessments to advance ecological impacts (*4*). Chemical industries often criticise the early tiers as being too conservative, as fast dissipation and low bioavailability of residues in the field reduce the negative effects on organisms suggested by standard toxicological tests carried out in laboratories. For this reason, many companies finance ecological studies to demonstrate the administrators that the actual impacts of their chemicals on the environment are acceptable under normal practices (*5*).

Probable causes of discrepancy between the early tiers of the assessment, usually based on the standard hazard quotient (*6*), and more advanced ecotoxicological studies stem from: i) inadequate extrapolation of toxicity data from standard test-species to local species, which often do not have the sensitivity of the surrogates (*7*); ii) unrealistic concentrations of residues in water, soil or vadose zone and air, usually higher than observed in field situations; and iii) omission of physical and chemical properties in the mathematical models of the assessment, which leads to gross misinterpretation of the actual levels of toxicants with time and their bioavailability in complex ecosystems.

The first cause has been addressed sufficiently by researchers during the last decade (*8, 9*). Perhaps as a result of these studies, the probabilistic method for risk assessment (*10*) emerged as a solution to the problem. However, this method is applicable with one chemical at a time, not with mixtures, and involves using large sets of data which are often lacking when new chemicals are to be registered. The second cause, excessively high residue concentrations, may be partly justified in order to establish safety levels that protect either human health or the environment, the so-called worst case scenario. Nevertheless, it is equally reasonable to assess the chemical impacts using realistic concentrations that can be compared with the worst case scenario so that a more balanced judgement can be achieved (*11*), as it is usually done in the second tier of the assessments. But it is the third cause that arises more concern on the part of environmental scientists. Indeed, the hazard quotient alone does not consider the fate of residues with time, despite its reality, nor their bioconcentration in the organisms exposed. Such quotients only refer to a group of organisms (taxon) closely related to the surrogate species with which they are compared, irrespective of whether they are or not the common species found in a particular ecosystem.

To solve the inadequacy of the current assessment methods, at least in regard to impacts on ecosystems, we proposed the Ecological Relative Risk (EcoRR) as an alternative (*12*). Because of its novelty, this method is still at the evaluation stage, so we decided to carry out some outdoor experiments with several chemicals in an effort to understand the meaning of the EcoRR scores, a kind of introspective study or self-appraisal. At the same time, the hazard quotients were calculated and contrasted with the EcoRR scores to obtain a comparative assessment of the fitness of both models to the reality.

Any validation procedure requires a field dataset, which is then subjected to analysis using the various mathematical models available. To this purpose, the results of a two-year monitoring study on small experimental rice fields (mesocosm) were used as a case study. The steps required to obtain and analyse relevant field data for validation of hazard quotients and EcoRR scores are explained here.

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## Validation of Risk Methodologies Using Field Data

Mesocosms are useful designs for validation of risk models because they allow detailed sampling and monitoring of almost all species present, while the researcher can choose the conditions, chemicals and other variables at will. As control and treatments mesocosms are subjected to the same environmental factors, one source of error (i.e. environmental variability) is reduced to a minimum. Although mesocosms are not representative of natural ecosystems as such, they can be very useful to test the validity of risk assessment methods, provided they simulate adequately the ecosystems of reference. The following sections illustrate a case study as a guide to show how this can be achieved.

### Experimental Design

Six small experimental fields (5.2 x 1.6 m) located at the National Institute for Environmental Sciences, Tsukuba (Japan), were planted with rice and monitored during two different seasons. In the first year (2004), two fields were treated with the systemic insecticide imidacloprid (Admire GR, 21.5 kg $ha^{-1}$), two others with an anti-dandruff shampoo containing the active ingredient zinc pyrithione (Zpt, 7.3 L $ha^{-1}$), and the remaining two fields were kept as controls, i.e. no chemical treatment of any kind. In the second year (2005), another imidacloprid treatment (15 kg $ha^{-1}$) was applied on the same two fields as the previous year, which were in turn treated three months later with the insecticide etofenprox (75 L $ha^{-1}$). Two more fields were also treated with etofenprox alone at the same rate, leaving the remaining two fields as controls.

All fields were flooded with groundwater to a depth of approximately 4 cm a few days before rice transplanting, which took place on May 14, 2004 and April 27, 2005 respectively. Each field had an array of 27 x 3 rice swards, leaving a 15 cm embankment on each side (bunds), and remained flooded until the end of monitoring on September 16, 2004 and September 1, 2005 respectively. Water from the fields drained to individual culverts located at the bottom end, where it was recycled using an automated pumping system that kept water levels constant in the paddies, and ensured that each field remained independent in regard to water conditions and chemical residues.

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## Choice of Chemicals

The chemicals chosen in this case are insecticides commonly applied to protect rice crops in Japan and other countries (imidacloprid, etofenprox), and a toxic pollutant (Zpt) which may find its way into paddy irrigation waters (*13*). Their different physical and chemical properties, toxicology and mode of application (Table I) make them ideal for comparing their ecological risk.

Imidacloprid is a water soluble, systemic neonicotinoid insecticide, which undergoes fast photolytic degradation. Its prolongued effectiveness (*14*) is counterbalanced by a low rate of application in rice paddies (150 g a.i. $ha^{-1}$), thus making it unlikely to affect zooplankton and insect larvae; besides its toxicity to fish and vertebrates is low (Table II). Etofenprox is a non-ester pyrethroid insecticide of very low solubility in water and high adsorption onto organic matter. It is very toxic to aquatic crustaceans and fish, but its field half-life is short. Finally, the pollutant Zpt is insoluble in water and dissipates extremely fast in the sunlight. However, it is the most toxic of the three selected chemicals, to aquatic invertebrates and vertebrates alike.

## Monitoring of Chemicals in Water and Soil

Duplicate samples of water (500 mL) and surface wet soil (2-3 cm, approx. 250 $cm^3$) from the treated fields were taken at time intervals starting at 2 h after application - or transplanting of seedlings in the case of imidacloprid -, followed by sampling at 4 days, one week, two weeks and monthly thereafter until the end of the four-month study period.

For chemical analysis of imidacloprid residues in water and soil, the method of Baskaran et al. (*15*) using HPLC was followed. Residues of etofenprox

**Table I. Physical and chemical properties of the three chemicals used in this study (*21, 25, 30*), and their application features.**

| | *Etofenprox* | *Imidacloprid* | *Zpt* |
|---|---|---|---|
| Molecular weight | 376.5 | 255.7 | 317.7 |
| Solubility in water (mg $L^{-1}$) | 0.001 | 610 | Insoluble |
| Vapour pressure (mPa) | $9.1 \times 10^{-4}$ | $4 \times 10^{-7}$ | Not available |
| Partitioning coefficient (log $K_{ow}$) | 7.05 | 0.57 | 9.33 |
| Bioconcentration factor (BCF)* | 6.90 | 1.12 | 8.94 |
| Half-life in water | 7.4 days | 2 h, 4 days | 25 min, 4 h |
| Half-life in soil or sediment | 15 days | 50 – 70 days | 0.5 h (anaerobic)<br>2-22 h (aerobic) |
| Mode of application | Spray | Seedlings in nursery box | Shampoo in water |
| Application rate (L $ha^{-1}$) | 75 | 21.6 (2004)<br>15.2 (2005) | 7.3 |
| Active ingredient (%) | 0.1 | 1.0 | 1.0-2.0 |

* BCF = 0.607+0.893 log $K_{ow}$ (*29*)

in water were extracted with methanol to give 40% v/v, followed by SPE with $C_{18}$ cartridges, using the method of Nakamura et al. (*16*), and the etofenprox contents analysed using GC-MS. Residue levels of the chemicals in rice plants or adjacent weeds could not be determined analytically in this study: instead, estimates of the highest possible residues of imidacloprid and etofenprox in rice plants were calculated by subtracting the amounts already found in water and soil from the total amount used. In the case of Zpt, nominal concentrations at the time of addition (5 mL twice a week) were deemed more reliable for the purpose of risk assessment than actual measurements. Indeed, the standard method of extraction from water shows low recoveries (*17*), while no established method for residues in soil is currently available, perhaps due to its strong chelating properties (*18*).

## Monitoring Aquatic and Terrestrial Communities

Ecological risk assessmenst focus on the overall impacts that chemicals have on the communities of plants and animals that live in an ecosystem. This is reasonable, since any negative effect would lead eventually to a population decline, or else the effects are not 'negative' in the ecotoxicological sense (*19*). Consequently, to evaluate the ecological risk of the three chemicals used in this study, population changes of all species present in the rice mesocosms were monitored throughout an entire four-month cultivation period of this crop.

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**Table II. Median lethal toxicity endopoints (ppm) for the three chemicals studied**

| *Taxon* | *Surrogate species* | *Endpoint* | *Etofenprox* | *Imidacloprid* | *Zinc pyrithione* |
|---|---|---|---|---|---|
| Mammals | Mouse | Acute oral LD$_{50}$ | $107 \times 10^3$ (*30*) | 98 * | 160 * |
|  | Rat | Acute oral LD$_{50}$ | $42.8 \times 10^3$ (*30*) | 410 * | 177 * |
| Birds | Bobwhite quail | Acute oral LD$_{50}$ | - | 152 (*30*) | - |
|  | Japanese quail | Acute oral LD$_{50}$ | - | 31 (*30*) | - |
|  | Mallard duck | Acute oral LD$_{50}$ | >2000 (*30*) | - | - |
| Frogs | *Rana* sp. | 48-h LC$_{50}$ | - | 165 – 219 (*36*) | - |
| Fish | *Brachidanio rerio* | 96-h LC$_{50}$ | - | 281 (*37*) | - |
|  | Carp | 96-h LC$_{50}$ | 5.0 (48-h) (*30*) | 170 (*14*) | - |
|  | Golden orfe | 96-h LC$_{50}$ | - | 237 (*30*) | - |
|  | Medaka | 24-h LC$_{50}$ | - | - | 0.1 (*21*) |
|  | *Oreochromis niloticus* | 48-h LC$_{50}$ | 8.2 (*31*) | - | - |
|  | *Pimephales promelas* | 96-h LC$_{50}$ | - | - | 0.003 (*44*) |
|  | Rainbow trout | 96-h LC$_{50}$ | - | 211 (*30*) | 0.003 (*44*) |
| Insects | *Apis mellifera* | - | - | 0.018 (*38*), 0.6 | - |
|  | *Aphis pomi* | - | - | (*39*) | - |
|  | Cicadellidae | 24-h LC$_{50}$ | 5.3 – 72.1 (*32*) | 1.8 (*40*) | - |
|  | *Cyrtorhinus* | 24-h LC$_{50}$ | 17.2 (*33*) | - | - |
|  | *lividipennis* | - | 0.006 (*34*) | - | - |
|  | Culicidae larvae | 24-h LC$_{50}$ | 0.03 (*31*) | - | - |
|  | Ephemeroptera | 48-h LC$_{50}$ | - | - | - |
|  | *Hyaliodes vitripennis* | - | - | 2.0 (*28*) | - |
|  | Orthoptera | - | - | 53-86 (*41*) | - |
| Crustaceans | *Caridina africana* | 24-h LC$_{50}$ | 0.03 (*31*) | - | - |
|  | *Daphnia* sp. | 48-h LC$_{50}$ | >40 (3-h) (*30*) | 10 (*42*), 85 (*30*) | 0.034 – 0.1 |
|  | *Hyalella azteca* | - | - | 0.055 (*43*) | (*44,27*) |
|  | Ostracoda | 48-h LC$_{50}$ | - | 0.3-0.7 (*27*) | 0.1 – 0.5 (*27*) |
| Invertebrates | *Chironomus* sp. | 48-h LC$_{50}$ | 10.5 – 23.2 (*35*) | 1.6 – 9.8 | - |
|  | *Crassostrea virginica* | 96-h LC$_{50}$ | - | - | 0.022 (*44*) |
|  | Earthworms | 7-d LC$_{50}$ | 43.1 (*30*) | 10.7 (*30*) | - |

* ChemIDPlus database (http://chem.sis.nlm.nih.gov/chemidplus/)

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In small mesocosms, species populations can be estimated with reasonable accuracy from direct counts of individuals sighted or sampled at a specific place and time, in spite of the sampling errors inherent to the various techniques (*20*). Large organisms like birds or mammals could not be included in the study, while microscopic organisms living in soil, water (phytoplankton) and plants were also excluded; therefore, only small organisms such as medaka fish, frogs, arthropods and other macro-invertebrates of the paddy ecosystem were monitored. All monitoring was done on a weekly basis, though it was more intense in the week after transplanting and following the application of etofenprox.

A total of 90 species were monitored in the first year and 112 in the second, with 60% of them being aquatic. Among these, nine species of insects are considered pests of rice crops (*13*). The remaining can be regarded as non-target species for the insecticides applied, while all aquatic organisms were subjected to their effects and those of the pollutant Zpt.

Typically, a rice field is composed of five communities: plankton, neuston (water surface organisms), nekton (aquatic insects and their larvae, fish and tadpoles), benthos (bottom dwelling organisms), and crop arthropods.

For each community, a specific sampling or census technique is required given the obvious differences in body size and abundance of the pertaining species. Thus, zooplankton crustaceans and small aquatic organisms were sampled using a polyvinyl chloride (PVC) core (15 cm height, 10 cm diameter) that was placed at three random spots in each field. Water in the core was sucked using a manual pump, filtered through a 250 μm mesh and the contents placed in 20 mL vials. Approximately 2-3 cm of topsoil under the PVC cores was removed using a perforated scoop, and placed in plastic containers. Large aquatic insects, crawling larvae and other invertebrates were thus collected by washing the soil through a 0.85 mm sieve.

A number of medaka fish (*Oryzias latipes*) were released in each field on transplanting day of either year (*21*), and their adult population was monitored weekly for as long as the crop plants allowed accurate counts. In the case of the crop arthropods, all individuals seen in the rice crop and adjacent bunds, mainly insects and spiders, were counted.

## Data Analysis

Both the hazard quotient and the Ecological Relative Risk methods are based on the standard framework for risk assessment (*22*), which estimates risks by comparing the exposure levels of toxicants (i.e. concentrations) against a relevant toxicological endpoint of reference, usually the $LC_{50}$ or $LD_{50}$ of the species concerned. In this case study, the highest concentrations of chemicals and their available toxicological data were used with either method: the specific parameters used are presented in the respective sections below. The disparity of species compiled in the toxicological dataset (Table II) justifies the use of geometric means for all the species in a taxon, group of organisms or community for which the assessment is made (*23*).

Obviously, the hazard and risk values thus obtained refer to the worst case scenario, since dissipation of chemical residues certainly occur in any ecosystem. Although this point may seem irrelevant for comparing methods, as any other set of concentrations would do equally, it is very relevant to the validation procedure intended here because it means that the observed negative effects are undoubtedly due to lower concentrations of chemicals than those used in the desktop assessment, and therefore there is no room for complacency in the model's results.

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## Observed Ecological Effects

A detailed account of the first year's results can be found in (*13*), whereas those for the second year are shown here for the first time. For ease of presentation in the following figures, population densities from the controls were subtracted from each of the respective treatments, while the percent reduction or increase of each treatment and their statistical significance are indicated.

### Zooplankton Community

Populations of zooplankton in untreated paddy fields showed a steady increase from almost zero levels at transplanting day to densities of 100-300 organisms $L^{-1}$ after a month or two. Fields planted with imidacloprid-treated seedlings were almost devoid of zooplankton for the first two months (Figure 1). During that time, concentrations of imidacloprid in water decreased from 239-193 $\mu g\ L^{-1}$ to $1.0 \pm 0.7\ \mu g\ L^{-1}$. Zooplankton species in these fields did not recover until mid-July, and when they did attained lower densities than in the controls. Such significant effect ($P<0.001$) cannot be explained other than by direct toxicity of this chemical, whose activity in paddies is known to last two or three months (*14*). Contrary to our expectations, no significant difference was found between zooplankton in the fields treated with 36 regular inputs of Zpt-shampoo and that from the controls. Lack of effects are most likely due to insufficient exposure to this pollutant, which residue levels in water would be below the detection limit (0.02 $\mu g\ L^{-1}$) (*17*) in one or two days.

The effect of etofenprox on zooplankton could not be determined as control populations collapsed for unknown reasons — though the presence of a predatory worm (*Aelosoma* sp., Aphanoneura) may have caused it.

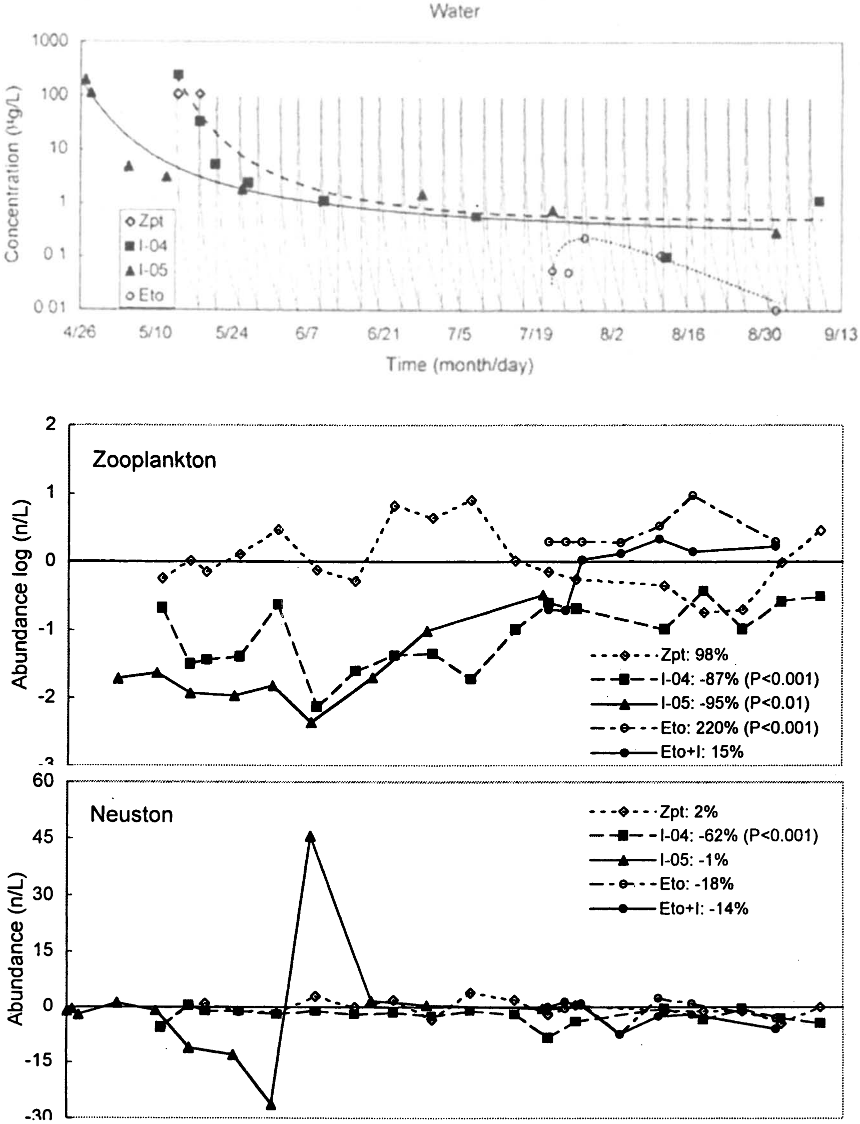

*Figure 1. Concentrations of zinc pyrithione (Zpt), imidacloprid (I-04, I-05) and etofenprox (Eto) in water of a rice mesocosms (top), and their impact on the zooplankton, neuston and nekton communities.*

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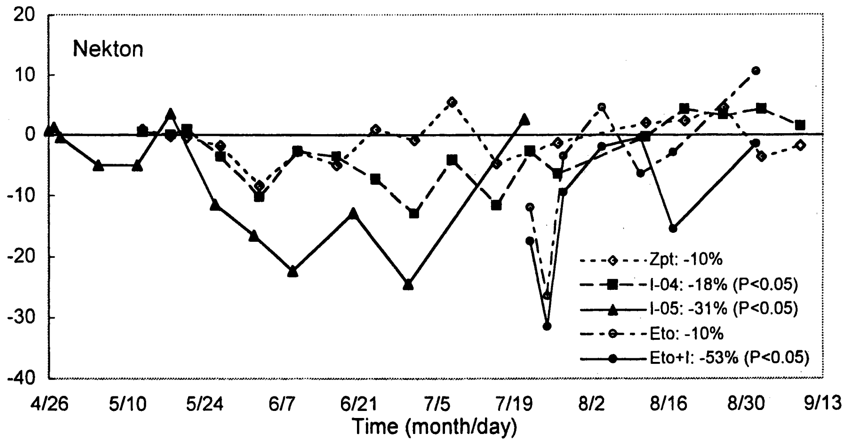

*Figure 1. Continued.*

Concentrations of etofenprox in water in the first week were 0.06-0.23 µg $L^{-1}$, which possibly account for its lack of impact in view of the current toxicological data. In summary, the direct negative ecological effects of the three chemicals can be ranked imidacloprid >> etofenprox = Zpt. Indirect effects were also detected as a consequence of the absence of zooplankton in imidacloprid fields (*13*), but they are outside the scope of this paper.

## Macro-invertebrate Communities

The 115 species of arthropods, three worms and one snail monitored during the two years were studied in four separate communities, as indicated above. Population densities of the neuston community (11 insect species and three spiders) were generally low in controls as well as treated fields. Statistical differences were, therefore, difficult to detect, excepting imidacloprid in the first year (62% reduction, $P<0.001$). Fields treated with etofenprox had 14% to 18% lower densities than the control fields, whereas Zpt produced an insignificant 2% increase (Figure 1) Results for imidacloprid treatment in the second year, however, were hampered by a sudden increase in young waterstriders (*Gerris paludum*) in June, which offset the previous decline (-26%) of this community. In summary, imidacloprid affects significantly the early stages of the neuston community, when many waterstriders and other insects are found immobilised on the water surface of the paddy by being directly exposed to concentrations above 1 µg $L^{-1}$; etofenprox does not have significant impacts because the timing

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of its application coincides with the absence of most of the species in this community; and Zpt has no impact at all in spite of its regular input into the aquatic system.

The nekton community included 10 species of aquatic insects and 17 others whose larvae are also aquatic: dragonflies, mayflies, mosquitoes and caddisflies. It must be said that all these organisms are non-target species, mostly predators which control some pests of rice on the spot, so negative impacts on this community are very important. Aquatic arthropods in the control fields increased rapidly in the first month and after some fluctuations reached their highest densities in July (18-35 organisms $L^{-1}$), declining afterwards because many larvae emerged into adult insects. Imidacloprid residues in water during the first two months obviously affected most aquatic larvae even if residue levels were as low as 1 $\mu g\ L^{-1}$ after one month. Thus, a 46-59% reduction was measured for the June-July period. As differences between these fields and the controls became smaller in the subsequent months, the overall impact was reduced to a significant ($P<0.05$) 18% and 31% for 2004 and 2005 respectively (Figure 1).

Just when the larval populations were recovering in the imidacloprid-treated field by the middle of the season the etofenprox application pushed them back again, but this time reducing them by 91% in one week. The etofenprox application alone reduced the nekton community by 69% in the first week, which may indicate that the combined effect of two insecticides applied consecutively had a greater impact than one insecticide alone in the season (*24*). In both cases the effect of etofenprox was significant ($P<0.05$) but the community recovered in two weeks. Surprisingly, these effects took place when concentrations of etofenprox in water were below 0.2 $\mu g\ L^{-1}$, which is far less than its lowest $LC_{50}$ known for insect larvae. Again, no significant population changes were observed among aquatic arthropods of the Zpt-shampoo treated fields, though their overall densities were 10% lower than in control fields. Comparatively, ecological effects of the three chemicals on the nekton arthropod community are in the following order: etofenprox > imidacloprid >> Zpt.

The benthic community (five species of insect larvae, three worms and one snail), was affected mostly by residues of the chemicals in soil and to a certain extent by those in the water phase infiltrating the paddy soil (approx. 40% of total mass). Control fields showed a large population of chironomid larvae in the first month, later decreasing to levels 10 to 20 times lower, while snails increased in the late season. In the first month, imidacloprid-treated fields experienced significant reductions in density: 98% ($P<0.01$) and 67% ($P<0.05$) in the first and second years respectively. This effect occurred while concentrations of this insecticide in soil were 2-9 $\mu g\ kg^{-1}$ (dry wt) in the first year and 0.7-2.1 $\mu g\ kg^{-1}$ in the second. Zpt-shampoo treated fields suffered a 44% reduction in the number of chironomids ($P<0.05$, Figure 2). However, no effects of either imidacloprid or Zpt were observed later in the season, indicating that neither chemical accumulated in the system. The etofenprox application, on the

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other hand, did not alter this community in the least: a non-significant 5% reduction was observed in the fields under etofenprox alone and a 23% increase in those with imidacloprid-treated rice; the latter result is difficult to explain. The toxicity of this insecticide to chironomids and worms is relatively low, with $LC_{50}$s three orders of magnitude higher than the residues in paddy soil (4-31 μg $kg^{-1}$). In summary, observed impacts of the three chemicals on the benthic communities are: imidacloprid > Zpt >> etofenprox.

Arthropods on the crop (46 insects and eight spiders) included 11 species of insects that spent most of their developmental stages in the water (e.g. dragonflies) and moved to the crop later, in their adult stage. All pest species belong to this community exclusively. As with zooplankton, crop arthropods experienced fluctuations in density due mainly to replacement of one species for another during the rice season and to changes related to their life cycles.

It should be noted that imidacloprid fields treated at the highest rate had significantly lower populations ($P<0.01$) than control fields, whereas overall differences in the second year did not have as much an impact (Figure 3). The low peak in density observed about a month after transplanting corresponds to the insecticidal activity on the rice weevil (89% and 95% reduction for 2004 and 2005 respectively), which was recorded in detail for being one of the target species of this insecticide. The application of etofenprox later in the season was felt only on fields previously treated with imidacloprid, where populations suffered a small but significant impact in the first week ($P<0.05$), after which time the community appears to have recovered quickly. It seems that etofenprox alone did not affect much the arthropod populations: a mere 3% reduction, insignificant by all accounts. The overall negative effects of these insecticides on the non-target community was slightly higher in the case of imidacloprid than etofenprox.

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## Vertebrate Community

The above ecological impacts are more noticeable for insecticides on invertebrate communities because such chemicals are designed to kill insects. In contrast, concentrations of imidacloprid in water during the first two months (from 240 to 1 μg $L^{-1}$) had no effects on populations of vertebrates such as tadpoles or medaka. However, judging by the sublethal effects of imidacloprid observed in medaka fry born in the fields (*21*), it is likely that all vertebrates of the nekton community had felt the effects of chemical toxicity to some extent.

In this regard, the most remarkable impact was medaka direct mortality, which reached proportions of 25% in the first week after treatment with Zpt-shampoo, but not afterwards in spite of the repeated addition of this product during four months, suggesting that the surviving fish were tolerant to the

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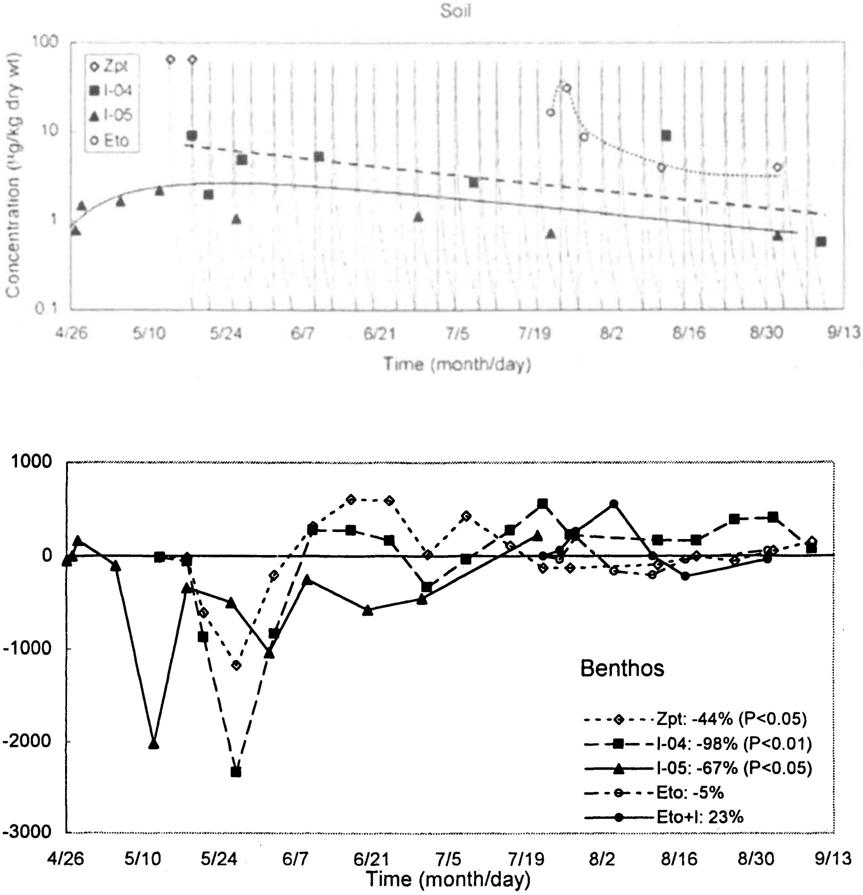

*Figure 2. Concentrations (μg kg$^{-1}$ dry wt) of zinc pyrithione (Zpt), imidacloprid (I-04, I05) and etofenprox (Eto) in soil of the rice mesocosms and their impact on the benthic community of the ecosystem.*

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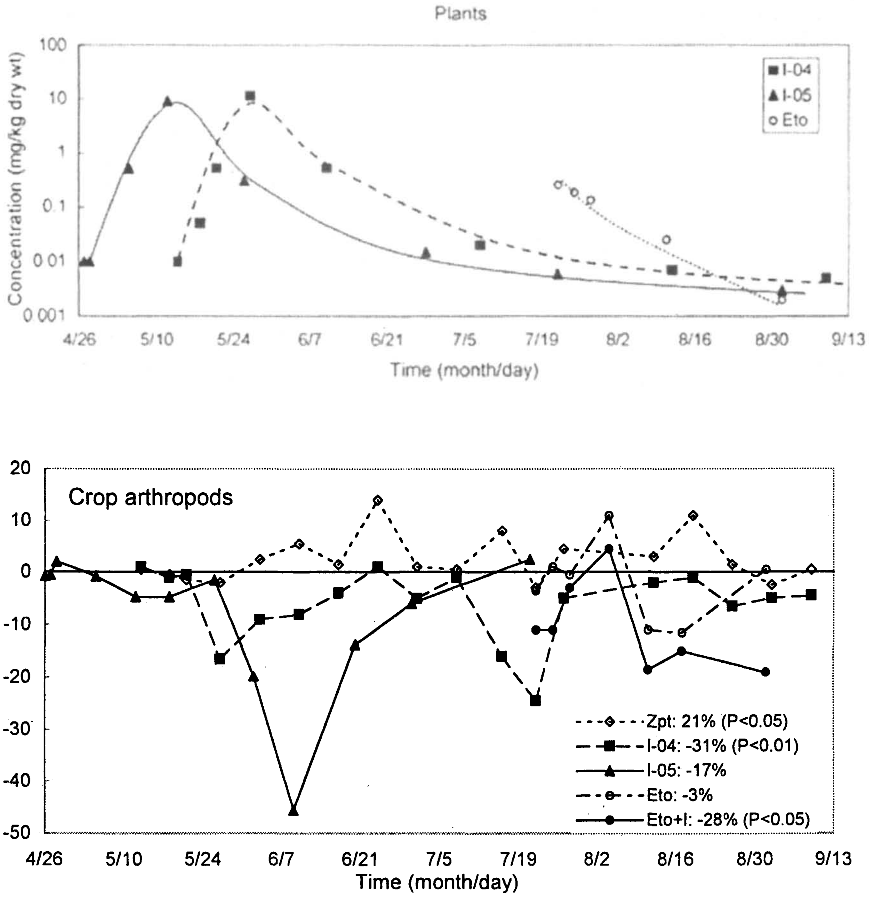

*Figure 3. Estimated concentrations (μg kg$^{-1}$ dry wt) of imidacloprid (I-04, I05) and etofenprox (Eto) in plants of the rice mesocosms and their impact on arthropods of the rice crop.*

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weekly concentrations added (*21*). A similar proportion of adult medaka (30%) was found dead the day after etofenprox was applied, but only on the fields planted with imidacloprid-treated seedlings, whereas no deaths were recorded on fields that had no previous insecticide treatment. Given the low concentrations of etofenprox measured in water and sediment the day after spraying (0.055 and 16 ppb for the respective media), this observation is surprising considering that $LC_{50}$s of this insecticide to other fish are between 5 and 8 mg $L^{-1}$. By contrast, medaka losses on imidacloprid-treated fields were only 2-5% in both years, in agreement with a low toxicity of this insecticide to fish.

As for amphibians, soon after the commencement of the experiment frogs came to spawn, and thereafter visited the paddies from time to time. The numerous tadpoles that hatched there thrived in all fields regardless of the treatment imposed, and no signs of mortality were observed at any time; but once they became adult frogs they migrated elsewhere, thus causing the decline of their paddy populations. For vertebrates, therefore, relative impacts of the chemicals used are ranked as follows: Zpt = etofenprox + imidacloprid >> imidacloprid > etofenprox alone.

## Matching Observations with Predicted Impacts

Concentrations of insecticide in the mesocosms varied throughout the study period, but only the highest values measured in water and soil were used in the risk models: values for imidacloprid were 0.19 or 0.24 mg $L^{-1}$ in water, and 2.2 or 12.8 μg $kg^{-1}$ in soil depending on the year, and for etofenprox were 0.23 μg $L^{-1}$ and 31 μg $kg^{-1}$ in water and soil respectively. For Zpt, the highest possible residues were obtained through modelling (*25*): 54 μg $L^{-1}$ in water and 32 μg $kg^{-1}$ in soil after each addition of 5 mL anti-dandruff shampoo, considering that no accumulation of residues took place. Estimates of insecticide concentrations in the crop plants rendered values of 9.8 and 13.5 mg $kg^{-1}$ for imidacloprid in either year, and 0.25 mg $kg^{-1}$ for etofenprox. Such estimates are consistent with measurements reported by other authors (*14*).

### The Hazard Quotient Method

When using acute toxicity data, values of HQ above 1 are considered unacceptable in environmental assessments, and quotients above 0.1 are regarded with concern because populations may be decimated, i.e. suffer 10% or more losses by direct mortality (*9, 26*). Only values of HQ <0.1 are, therefore, acceptable.

Five groups of organisms were considered in this case: aquatic vertebrates, aquatic arthropods, arthropods of the crop, zooplankton and benthic organisms. In the latter group the quotient was calculated twice, using the exposures in water and soil, as such organisms are exposed to both media (Table III).

**Table III. Hazard quotients for the three toxic chemicals studied, using the highest concentrations in water (mg $L^{-1}$), soil and plants (mg $kg^{-1}$ dry wt).**

| | *Etofenprox* | | | *Imidacloprid** | | | *Zpt* | |
|---|---|---|---|---|---|---|---|---|
| | Water | Soil | Plants | Water | Soil | Plants | Water | Soil |
| Concentration | $2 \times 10^{-4}$ | 0.031 | 0.25 | 0.24<br>0.19 | 0.013<br>0.002 | 13.6<br>9.8 | 0.054 | 0.032 |
| Vertebrates | <0.01 | - | - | <0.01<br><0.01 | -<br>- | -<br>- | 2.89 | - |
| Arthropods | <0.01 | - | 0.06 | 0.15<br>0.12 | -<br>- | 8.67<br>6.23 | 1.67 | - |
| Zooplankton | <0.01 | - | - | 0.04<br>0.03 | -<br>- | -<br>- | 0.32 | - |
| Benthos | <0.01 | <0.01 | - | 0.09<br>0.08 | 0.01<br><0.01 | -<br>- | 1.90 | 1.13 |

The geometric mean $LC_{50}$ for species in each group (Table II) was used in all cases.

* Upper values for 2004 and lower values for 2005

According to the quotients, imidacloprid would be the most hazardous of the three chemicals to arthropods of the crop (HQ = 8.67 and 6.23), inflicting severe losses on populations of many beneficial predatory insects and spiders. However, our experimental data contradict this assessment, since the particular mode of application of this insecticide restricts its activity to sucking insects (nine species in our study) and borer larvae (five species), which constitute most of the pest species of rice crops, leaving all other arthropods practically unharmed. Quotient values of 0.15 and 0.12 were obtained for the aquatic arthropods, but predators such as waterstriders and larvae of dragonflies and beetles certainly suffered more the effects of this insecticide (18-62% reductions) than the HQs indicate. With regard to the zooplankton and benthic communities, imidacloprid quotients appear to underestimate the real impacts, since the almost complete elimination of zooplankton in the first two months after transplanting is at odds with HQ values of 0.04 and 0.03 for either year.

Similar values were obtained with the benthic chironomids (HQ = 0.09 and 0.08), although in this case the effect was specific (*C. yoshimatsui* was absent for two months) and the community recovered soon after imidacloprid residues in

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water and sediment were below 1 μg $L^{-1}$. It is also obvious that the toxicological data available for imidacloprid do not represent adequately the species found in these two rice paddy communities.

Etofenprox hazards are estimated as low for non-target arthropods on the crop (HQ = 0.06), which receive most of the insecticide spray, and this is confirmed by the data collected on some fields (3% reduction) whilst those treated previously with imidacloprid showed higher impacts (28% reduction). No other impacts could be forecasted using the available toxicological data, yet in reality the aquatic arthropods and medaka fish suffered reductions of 18-69% and 30% in their respective populations. This unpredictable outcome may have resulted from magnification of etofenprox residues in particulate organic matter – including zooplankton - present in paddy water, which make up the diet of such organisms.

The most striking mismatch occurred, however, when trying to assess the ecological impacts of the pollutant Zpt using the hazard quotient: most values are above 1, indicating serious impacts for vertebrates, aquatic arthropods, larvae and benthic organisms, whereas in reality significant population reductions only occurred among chironomid larvae (44%) and medaka fish (25%), in the latter species at lower levels than expected by its own $LC_{50}$. Hazards on zooplankton are also overestimated (HQ = 0.32) as neither changes in species nor their populations were observed at all. Such blunders cannot be explained by flawed toxicological data alone, but rather by a deficiency of the quotient method itself, which does not consider the dissipation features and physico-chemical properties of degradable compounds.

## Ecological Risk Assessment Using EcoRR Scores

The major drawback of the hazard quotient is that no consideration is given to physical, chemical and environmental fate of the toxicants under assessment, which inevitable lead to false predictions, and hence the quotient usually overestimates the hazards (*11*). Many environmental scientists think this is a minor problem that can be corrected by accurately measuring the actual residues in water or soil, but the results of this study indicate that HQ predictions can be very misleading even if such measurements are known. The problem lies in the method itself, not in the accuracy of the residue data obtained.

For this reason, the ecological relative risk (EcoRR) method was proposed (*12*). Indeed, this method considers the same framework for risk assessment as the hazard quotient, i.e. exposure versus toxicity endpoints, but its mathematical expression includes modifiers of the exposure, while the average toxicity to the ecosystem takes into account the overall biodiversity. The EcoRR scores are

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calculated independently for each environmental compartment, and are added later to produce an overall score for the whole ecosystem.

Table IV summarises the EcoRR scores for water, soil and plant communities for the three chemicals tested here, using their corresponding physical, chemical and toxicological properties listed in tables I and II.

**Table IV. Risk scores ($EcoRR_C$) for three chemicals in water, soil and vegetation of a rice ecosystem.**

| | *Etofenprox* | *Imidacloprid** | *Zpt* |
|---|---|---|---|
| Crop | Very high (6,509) | Very high (15,363)<br>Very high (20,703) | - |
| Water | Negligible (0.1) | Low (1.3)<br>Low (1.1) | Moderate (134) |
| Soil | Moderate (130) | High (1,190)<br>Moderate (295) | Moderate-high (460) |

* Upper values for 2004 and lower values for 2005

According to this method, etofenprox risk was negligible in the aquatic environment, just as its HQ suggested, but such result clashes with the high impacts of this insecticide observed on aquatic arthropods and fish. Its EcoRR score in soil (130) indicates a moderate risk for the benthic community in agreement with our observations, whereas spraying above the crop results in a very high risk (EcoRR >6,000) which in reality was a mere 3-28% reduction. Possibly our surveys were unable to detect population effects due to movement of insects from adjacent control paddies and beyond. Truly, the extent of the arthropod mortality caused by this insecticide could not be assessed properly.

EcoRR scores over 15,000 indicate a very high risk of imidacloprid on crop arthropods of rice paddies, and high for the benthic communities (EcoRR >1,000), whereas the risk was supposed to be very low for aquatic organisms (EcoRR = 1.1-1.3). Once again the assessment failed, in this case for overestimating the impacts on the former two groups and grossly underestimating them for the latter one. It appears that not only the toxicological data of imidacloprid for zooplankton species is inappropriate (*27*), but also the known $LC_{50}$s for insects do not represent the toxicity to nymphs and larvae, usually twice as susceptible as the adults (*28*).

Finally, the EcoRR scores for Zpt reflected well the impacts of this chemical observed in the paddies contaminated with anti-dandruff shampoo. Moderate risks were determined for the aquatic (EcoRR = 134) and moderate-high for the benthic (EcoRR = 460) communities, with the latter impacts matching the observations: i.e. 44% reduction in chironomids. The contrast between these

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EcoRR scores and the overestimated HQ values for this pollutant is striking, and highlight the importance of including physical and chemical data in the models.

## Evaluation and Conclusions

The shortcomings of current hazard and risk assessment methods in accurately predicting ecological impacts lie mainly in inadequacies inherent to the models, which cannot take into account all the factors that control the exposure of non-target organisms to hazardous chemicals. Main sources of error arise from insufficient understanding of the exposure mechanisms in aquatic environments, whose dynamism pose a challenge for risk modellers because it is directly related to the toxic-kinetics of the compounds. Indeed, the main ecological impacts were observed among populations of aquatic organisms, whether insects and their larvae, zooplankton or fish, and most of them were underestimated by the hazard quotient and EcoRR methods. The exception was Zpt, whose impacts were exagerated by HQs but were correctly predicted using EcoRR scores. The use of accurate physical, chemical and toxicological data is, undoubtedly, the reason of such success.

Failure of both methods to predict negative impacts of etofenprox on aquatic organisms may be due to exposure factors that were not considered in the models: biomagnification of residues by aqueous organic matter surely enhanced acute toxic effects, even if the measured residue concentrations in the water phase alone are well below the $LC_{50}$ for most species. This is a major handicap for risk assessments of pyrethroid insecticides and other chemicals of similar behaviour. In the case of imidacloprid, available toxicological data for zooplankton species is evidently inadequate: current laboratory data cannot explain the almost complete absence of ostracods and cladocerans for the period of activity of this insecticide in rice paddies (*27*). Moreover, toxicological data of imidacloprid for insect larvae are still lacking, and the inclusion of $LC_{50}$s for adult insects such as bees and grasshoppers (Table II) leads to underestimations of risk by a factor of two or more.

Overestimates of risk for etofenprox and imidacloprid to non-target arthropods in the crop and adjacent sprayed areas should not be a concern: no large scale effects were apparent after spraying of etofenprox, while the specific mode of action of imidacloprid – affecting only sucking species - rules out impacts on most of non-target species of insects and spiders.

While acknowledging that no method is perfect, the hazard quotient seems inappropriate for assessments of fast degrading compounds such as Zpt, whereas the EcoRR model tends to overestimate impacts of insecticides on the non-target arthropod communities of the crop. In any case, the use of correct toxicological data is essential to make predictions of hazard or risk with these and other

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models. Since availability of toxicological data remains problematic, environmental researchers and chemical companies should make an effort to obtain relevant data that can be shared by scientists and regulators involved in ecological risk. Failure to provide such data only leads to misinterpretation of the actual risks to which organisms are exposed in natural environments.

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## Chapter 6

# Comparative Hazard Assessment of the Substances Used for Production and Control of Coca and Poppy in Colombia

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Glyphosate and an adjuvant, Cosmo-Flux® are employed for the control of coca and poppy plants used to manufacture the illicit drugs cocaine and heroin in Colombia, Latin America. Other substances, from pesticides to control pests in the coca and poppy fields to substances used in the extraction and refining processes are used by growers and refiners of the drugs. The practice of illicit crop production may have potential adverse effects on human and environmental health due to cut and burn practices and the large quantities of chemicals required to cultivate the crops under the conditions of growth in Colombia. Of the 67 substances used in significant quantities, 20 were selected as high hazard substances and 16 of these were pesticides. A comparative

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

approach was used to evaluate the relative hazard from glyphosate as used in the spray eradication program and the 16 pesticides used in the production of coca and poppy. Hazard quotients for the human indicated that several pesticides used in coca and poppy production present much greater hazard to humans than glyphosate. Hazard quotients calculated for the aquatic environments indicated that most of the pesticides used in coca and poppy production present significantly greater hazards to aquatic organisms than glyphosate (and Cosmo-Flux®). Several of the pesticides presented significant hazards to bees and other pollinators, however, the formulation of glyphosate plus Cosmo-Flux® was essentially non-toxic to honey bees. For the earthworm hazard assessment, only diazinon and carbendazim were more hazardous to earthworms than glyphosate.

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## Introduction

The growing and production of cocaine and heroin in Colombia has significant political, social, and economic implications as well as impacts on human heath and the environment. Coca (*Erythroxylum coca*) and related species are commonly associated with the tropical mountainous regions of South America with temperatures above 25°C and moderate to high rainfall >1,000 mm per year. Historically, coca played an important role in culture of the Incas, Quechuas, and many other Andean peoples. Cocaine, derived from the coca plant, is used in many countries as an illicit addictive drug; global production between 1995 and 2002 was estimated to range from 640 to 950 tonnes used by an estimated 14 million people (*1*).

Opium, morphine, and its derivative, heroin, are produced from the poppy, *Papaver somniferum*, which is primarily grown in Asia. Global production of opium in 2002 was estimated to be 1,586 tonnes, of which about 160 tonnes were produced in South America (*1*), some of this in Colombia. It is estimated that, globally, about 15 million people use opiates and that about 10 million of these use heroin (*1*).

Both coca and poppy are grown intensively in a process that involves the clearing of land, the planting of the crop and its protection against pests such as weeds, insects, and pathogens. Depending on the region, the clearing of the land for production purposes may have large and only slowly reversible effects on the environment. As for other forms of agricultural production, the clear-cutting of forests for the purposes of coca and poppy production reduces biodiversity, contributes to the release of greenhouse gases, increases the loss of soil nutrients, and promotes erosion of soils. As production is illegal, it normally takes place in remote locations that are close to or part of the Andean Biodiversity Hotspot (*2*). As a result, the clearing of land is done with little apparent consideration for the biological and aesthetic value of the ecosystem.

The growing of coca and poppy and the distribution of cocaine and opium/heroin in Colombia has been the focus of a National control and eradication program starting in the 1970s. The program involves a number of Departments and Agencies of the Colombian Government and is coordinated by the Direccion Nacional de Estupefacientes (DNE), an agency of the Ministry of the Interior and Justice. The program has three main foci; the control of production of coca and poppy through aerial spraying of the herbicide glyphosate; the control of the processing, purification, and transport of the cocaine and heroin; and the seizure and forfeiture of the profits of illicit drug production (*3*).

The aerial eradication program for coca and poppy in Colombia is the responsibility of the Antinarcotics Directorate of the Colombian National Police (DIRAN-CNP), supported by data gathering from other nations such those in North America and Europe. The DIRAN reviews satellite imagery and flies over growing regions on a regular basis to search for new coca and opium poppy growth and to generate estimates of the illicit crops through high resolution low-altitude imagery and visual observation. Flights with aircraft that spray coca and opium poppy crops with glyphosate are then conducted. Glyphosate is applied to coca at a rate of 4.9 kg a.e. per ha and to poppy at a rate of 1.2 kg a.e. per ha. An adjuvant, Cosmo-Flux® is added to the spray mixture to increase penetration through cuticular waxes (*4*).

Several concerns have been raised about the use of glyphosate and adjuvants in the eradication of coca and poppy plants. These concerns range from damage to other crops to adverse effects on the environment and human health. In response to this, the Government of Colombia appointed an independent environmental auditor who reviews the spray and no-spray areas with the DIRAN, and regularly monitors the results of spraying through field checks and analysis of data from the computer system. In addition to the internal assessment of the control program, three detailed reviews two on the substances used for production of cocaine and heroin (*5,6*) and one of the use of glyphosate (*4,7*) were conducted for the Inter-American Drug Abuse Control Commission (CICAD) section of the Organization of American States (OAS). These reviews form the basis for this Chapter which is an illustration of a comparative environmental and human health hazard assessment of the processes associated with the production and eradication of coca and poppy in Colombia.

Several pesticides are used in the production of illicit drugs (*7*). Herbicides may be used in the initial clearing of the land and later in the suppression of

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weeds. Similarly, insecticides and fungicides may be used to protect the illicit crops from pests and diseases. To increase yields, fertilizers and other nutrients may also be used. Large quantities of agrochemicals have been seized and confiscated as part of the program to control the production of illicit drugs (*3*). Although some of these agrochemicals are highly toxic to mammals and may have significant environmental impacts, accurate information on the amounts used, their frequency of use, and the conditions of their use is not available. Because of this, it is not possible to conduct a detailed human health and ecological risk assessment. In addition to the use of agrochemicals in the production of coca and poppy, large amounts of chemicals are used in the processing of the raw product into refined cocaine and heroin (*7*). Processing of the illicit drugs is conducted in remote locations and in the absence of occupational health and environmental regulations and controls. During and after use, these substances may be released into the environment and have significant impacts on human health and the ecosystem (*5*).

A total of 67 substances used in significant quantities for these purposes were reviewed in a Tier-1 assessment (*5*). From this list a detailed hazard ranking scheme was used to select the 20 most hazardous for a more detailed assessment of toxicological properties and their fate in the environment.(*6*). Of the 20, 16 were pesticides. Since no exposure data were available for any of the 16 pesticides, exposure estimates were conducted using the same procedures as were used for worst case estimates of glyphosate exposures during the aerial application of glyphosate and Cosmo-Flux® for the purposes of eradication of coca (*4*). This allowed the exposures to these pesticides to be compared to those of glyphosate as used in the eradication program. These hazards were then compared to those of other activities undertaken in the production of cocaine and heroin.

## Methods

### Pesticide exposures

#### *Humans*

Pesticides are applied with hand-operated backpack sprayers in coca fields (*6*). Formulated products are diluted with local sources of water from a nearby stream, river, or well. Mixing and loading of the sprayer usually takes place close to the water source and empty containers are discarded in the field. Other than anecdotal information, there are little data on the use of protective equipment; however, from field observations it appears not to be widely used.

As for the glyphosate risk assessment (*4*), the most likely scenario is the partially clothed human with a cross-sectional area of 0.25 m$^2$ exposed to the

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spray. For the purposes of this assessment, it was assumed that people conducting pesticide applications would be exposed via the same route as a bystander receiving an accidental overspray. However, this is likely an underestimate as an applicator would be handing concentrated material more often. In general, applicators have greater exposures than bystanders (*8*). Total body dose for each of the sixteen pesticides contained in the priority list was calculated from the pesticide application rate, dermal absorption of the pesticide, average human body mass, and surface area exposed. As for glyphosate, body dose calculations were computed using two different surface areas 0.25 m$^2$ (face, forearms, and hands) and 2 m$^2$ (face, hands, arms, feet, legs, and torso), which correspond to different clothing coverage scenarios. Pesticide absorption values (expressed as percent absorption) and application rates were obtained from government reports and the primary literature (references in *6*). Body dose was estimated from the equation:

$$Body\ dose = \frac{Application\ rate(mg/m^2) \times surface\ area(m^2) \times dermal\ absorption(\%)}{body\ mass(kg)}$$

### *Environmental*

As for the human exposures, similar procedures to those used to estimate surface water concentrations for glyphosate (*4*) were used to estimate concentrations of pesticides in water. The maximum concentration of pesticide water used for the hazard assessment of surface waters was estimated based on worst-case procedures, where direct overspray of water of different depths is assumed. Three assumptions of water depth were used, the USEPA assumption of a water depth of 2 m (farm pond *9*), the European assumption of a farm pond, 0.3 m, (*10*), and a depth of 0.15 m (forest pool or wetland). For an application rate of 1 kg per ha (1 x 10$^{-4}$ kg/m$^2$), the assumed maximum concentrations for these three depths are 50, 333, and 670 µg per L, respectively. These base values were adjusted by multiplying the assumed concentration at an application rate of 1 kg per ha by the suggested label rate in order to obtain specific exposure concentrations for individual pesticides.

Bees and other pollinating insects are important in agriculture and in the survival of many insect-pollinated plants. For this reason, they are tested for sensitivity to pesticides as part of the registration process. A general guideline has been suggested for assessing hazard of pesticides to honeybees (*11*). This is based on empirical observations in field tests with a number of pesticides. To use this, the rate (g AI) applied per ha of field is divided by the topical LD50 for the pesticide in µg per bee as determined in laboratory tests. The quotient is then compared to the hazard ratio criteria and the risk estimated. A hazard ratio of < 50 indicates low risk; 50 - 2,500 indicates moderate risk; and > 2,500 indicates large risk. Exposures to bees were determined from the recommended application rates. Concentrations of pesticides in soil were estimated using the assumptions based on a rate of application of 1 kg per ha to soil with a bulk density of 1.5 kg per L. For even distribution in the top 2.5 and 5.0 cm, this would give concentrations of 2.67 and 1.34 mg per kg soil, respectively. These values were adjusted for recommended application rates (*6*).

### *Human hazards*

The exposure value obtained from calculations divided by the effects value from experimental data, results in a Hazard Quotient (HQ). A HQ which exceeds one indicates a potential for toxicity; values less than one indicate toxicity is not likely to occur. For the human assessment, hazard quotients were computed by dividing the Reference Dose (RfD, obtained from the EPA IRIS database or other EPA sources) by the calculated body dose. The RfD (also known as the Acceptable Daily Intake or ADI) is a commonly-used criterion for judging exposure to a number of substances, especially pesticides. The RfD is the estimated maximum amount of an agent or pesticide, expressed on a body mass basis, to which an individual in a (sub) population may be exposed daily over their lifetime without appreciable health risk (*12*). This is used to assess chronic risk and therefore provides a conservative estimate of risk. It is the same estimator that was used to assess risks of glyphosate exposures that result from spray eradication (*4*) and thus serves as a useful criterion for comparative assessment of hazard. The data used in the calculation of the hazard quotients for humans are reported in (*6*). Toxicity and estimated exposure data for glyphosate in humans were included in for the purpose of comparison.

Most of the more hazardous pesticides (Figure 1) have hazard quotients (HQs) greater than 1, are insecticides, are toxic to mammals, and other wildlife, as well as to insects. It should be noted that the HQs are shown on a logarithmic scale to allow presentation in a small graph. These insecticides are organophosphorus compounds which are frequently associated with human poisonings and adverse effects in wildlife (*6*). The HQ for glyphosate was less than 1, as were those for carbendazim, cypermethrin, lambda cyhalothrin, and paraquat. Carbendazim is a fungicide and would not be expected to be hazardous to mammals. Cypermethrin and lambda cyhalothrin are pyrethroid insecticides, are moderately toxic to mammals, and are used at small rates of application. The small HQ for paraquat is reflective of its poor penetration through skin, the basis for the calculation of these hazards. In fact, paraquat can be much more hazardous if there are cuts or abrasions in the skin that facilitate penetration (*6*). If consumed orally, paraquat is very hazardous and is responsible for many human deaths, particularly where it is not used and stored properly (*6*).

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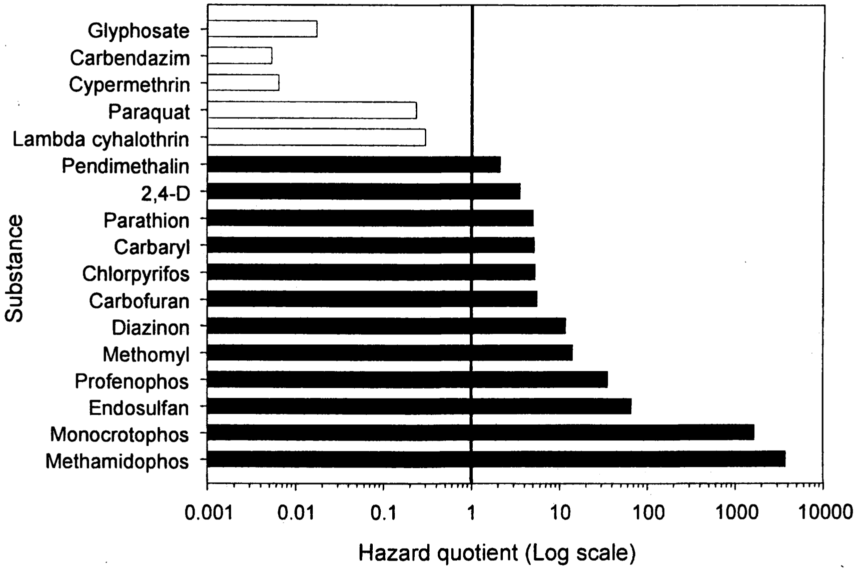

*Figure 1. Graphical illustration of the huma health hazard quotients of pesticides used in the production of coca and poppy. Quotients based on estimated body does as compared to the RfD.*

*Hazards to aquatic organisms*

The environmental HQ was calculated by dividing maximum estimated concentration in surface water by the lowest acute toxicity value for aquatic organisms (*6*). Again, this is a conservative estimate but is similar to that used for the assessment of the risk of glyphosate to non-target aquatic organisms (*4*) and allows for a comparative assessment of hazard. The hazard assessment data for exposures in 30 cm-deep surface water are shown in Figure 2. Toxicity and estimated exposure data for glyphosate and for the mixture of glyphosate and Cosmo-Flux® as used in Colombia are included for the purposes of comparison.

The hazard quotients calculated from environmental exposures in surface waters and the effect measure for the most sensitive aquatic organisms were also greater than 10 for several pesticides. In fact, for shallow waters (15 cm, data not shown), only pendimethalin and glyphosate (plus Cosmo-Flux®) had HQs less than 10. The HQ for endosulfan was, by comparison, 41,000 (*6*). Once again, most of the other pesticides used in the production of coca and poppy present a significantly greater hazard to aquatic organisms than glyphosate (and Cosmo-Flux®). Again, whether this represents a significant risk to the environment is uncertain as the frequency of use is not known. However, proximity of coca and poppy fields to surface waters is a constant with respect to

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use of pesticides by growers or eradication spraying from aircraft. Although not known exactly, the likelihood of contamination by pesticides used by coca and poppy growers and that from the use of glyphosate for eradication spraying is the same and these hazards can be used for compareative purposes.

## Hazards to bees

Several of the pesticides used in the production of coca and poppy have high hazard to bees, and by extension, to other pollinators (Figure 2). This is not surprising as these pesticides are insecticides and are very toxic to insects. Compared to these substances, glyphosate is essentially non-toxic to honey bees (Figure 2). Tests conducted with the formulation of glyphosate plus Cosmo-Flux® as used in the spray program in Colombia showed that it was also non-toxic to honey bees with no observed effects at exposures ≤ 58 µg per bee (*4*).

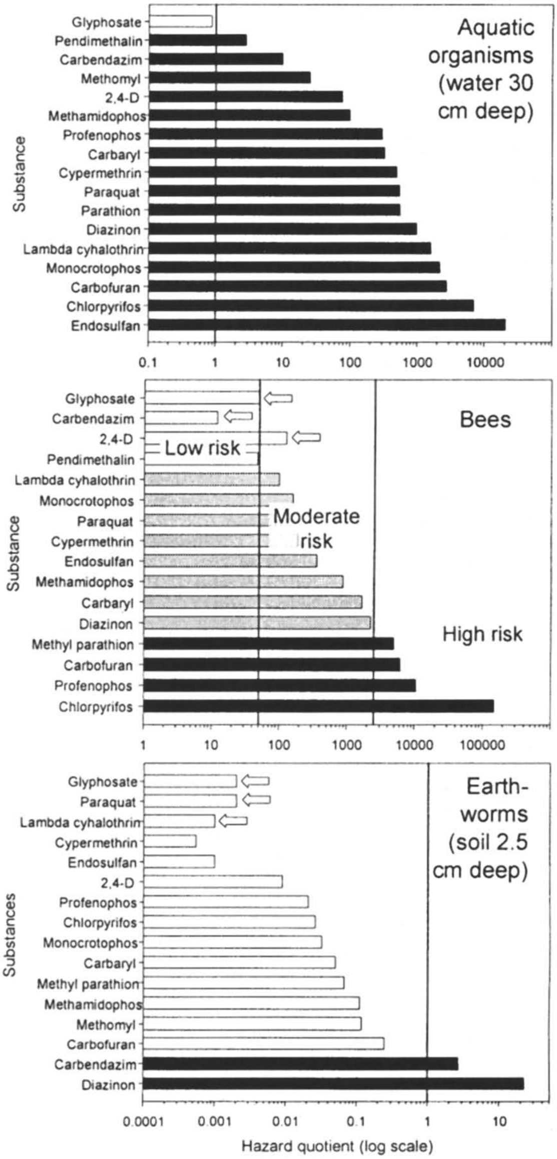

*Figure 2. Hazard quotients for aquatic organisms, bees, and earthworms for pesticides used in the production of coca and poppy. Arrows indicate that the HQs were based on toxicity data that were greater than the largest tested concentration.*

## Hazards to soil organisms

Soil organisms such as earthworms are important in maintaining soil quality and are routinely tested in the registration of pesticides. To assess hazards to earthworms, the data for the most sensitive soil organism (*6*) were compared to the concentration that would result if the soil was sprayed directly with the substance and it was evenly distributed in the top 2.5 or 5 cm of soil. Hazard ratios are shown in Figure 2. From these results, it is clear that a number of other pesticides that are used in the production of coca have greater hazards to earthworms than glyphosate. Diazinon and carbendazim both have hazard quotients greater than 1, suggesting that they may be hazardous to earthworms when used in coca and/or poppy production.

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## Comparative hazards from all activities in production and eradication of coca and poppy

There are a number of other activities associated with the process of cocaine and heroin production that result in risks to human health and the environment. While data were not available to quantify all the risks of these activities, some may be estimated on the basis of other knowledge and expert judgment. This was done using an adaptation of a risk prioritization scheme that has been used in ecological risk assessment (*13*). For the purposes of this ranking process for human hazards, the intensity score ranged from 0 to 5, with 5 being a severe effect such as a physical injury or toxicity. The recovery score also ranged from 0 to 5 and was based on the potential for complete recovery from the adverse effect. Frequency was based on an estimate of the proportion (%) of the total number of persons involved in coca and poppy cultivation, production, and the refinement of cocaine and heroin. The score for impact was the product of the individual scores and the percent impact is based on the sum of the impact scores (Table I).

Risks to humans and human health from the use of glyphosate and Cosmo-Flux® in the eradication of coca and poppy in Colombia were minimal (*4*). The acute toxicity of the formulated product and Cosmo-Flux® to laboratory animals was very small, the likely exposures were small, and the exposures were infrequent. When these risks are compared to other risks associated with clearing of land, the uncontrolled and unmonitored use of other pesticides to protect the coca and poppy, and exposures to substances used in the refining of the raw product into cocaine and heroin, they are essentially negligible. Compared to glyphosate exposures resulting from the eradication program, risks from potential misuse of and exposure to pesticides used in production were large.

A similar procedure to that described above was used for ranking ecological risks associated with the cycle of coca and poppy production. The intensity score was ranked from 0 to 5, with 5 being most intense, such as the total destruction of the habitat by clear-cutting and burning when clearing a natural area. Intensity of effects in this case also included off-field effects such as on non-target animals and plants.

**Table I. Potential human health impacts of activities in the cycle of coca or poppy production**

| *Activity* | *Intensity score* | *Recovery score* | *Frequency %* | *Impact score* | *% impact* |
|---|---|---|---|---|---|
| Clear cutting and burning | 5 | 3 | 3 | 45 | 16.7 |
| Planting the coca or poppy | 0 | 1 | 100 | 0 | 0.0 |
| Fertilizer inputs | 0 | 0.5 | 10 | 0 | 0.0 |
| Pesticide inputs | 5 | 3 | 10 | 150 | 55.6 |
| Eradication spray | 0 | 0 | 10 | 0 | 0.0 |
| Processing and refining | 5 | 3 | 5 | 75 | 27.8 |

Recovery time in this scheme is the estimated time for the impacted area to recover to a state similar to the initial condition. In the case of the clear cutting and burning, it is recognized that succession will begin immediately; however, full recovery to a mature and diverse tropical forest may take considerably more than the 60 years estimated here. Similarly, in the absence of cultivation, it was estimated that invasive and competitive species will displace coca and poppy in several years and an estimate of four years was used in this case. Given the need to apply fertilizer and pesticides frequently because of utilization of nutrients and resurgence of pests, the recovery time for these ecological impacts was judged to be small. The scores were multiplied to give the impact score and the percent impact was based on the sum of the impact scores (Table II).

Risks to the environment from the use of glyphosate and Cosmo-Flux® in the eradication of coca and poppy in Colombia were small in most circumstances (*7*). Risks of direct effects in terrestrial wildlife such as mammals and birds were judged to be negligible as were those to beneficial insects such as bees. Moderate risks to some aquatic wildlife may exist in some locations where shallow and static water bodies are located in close proximity to coca fields and are accidentally over-sprayed. However, when taken in the context of the environmental risks from other activities associated with the production of coca and poppy, in particular, the uncontrolled and unplanned clearing of pristine

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lands in ecologically important areas for the purposes of planting the crop, the added risks associated with the eradication spray program are small.

**Table II. Potential environmental impacts of the cycle of coca or poppy production**

| *Activity* | *Intensity score* | *Recovery time (y)* | *Impact score* | *% Impact* |
|---|---|---|---|---|
| Clear cutting and burning | 5 | 60 | 300 | 96.9 |
| Planting the coca or poppy | 1 | 4 | 4 | 1.3 |
| Fertilizer inputs | 1 | 0.5 | 0.5 | 0.2 |
| Pesticide inputs | 5 | 0.5 | 2.5 | 0.8 |
| Eradication spray | 1 | 0.5 | 0.5 | 0.2 |
| Processing and refining | 2 | 1 | 2 | 0.6 |

## Uncertainties

There were significant data gaps and uncertainties related to the rates of application, the frequency of the application, and the protective equipment used by the applicators working in coca and poppy fields. Additional uncertainties relate to other routes of exposure in bystanders and other workers who may re-enter the fields shortly after application of chemicals. Biomarkers of exposure, such as concentrations of pesticides and metabolites in urine and blood or inhibition of red blood cell acetylcholinesterase would be more appropriate indicators of exposure but are almost impossible to obtain for logistical reasons. A general uncertainty related to the use of chemicals in the refining and production of cocaine and heroin is the purity of these substances. In some cases, impurities may increase toxicity and hazard to humans and the environment.

Additional uncertainties result from some of the conservative assumptions used in the characterizing of exposures and toxicity. For environmental exposures, it was assumed that direct overspray of water or soil occurred. If surface water was not over-sprayed and the only contamination was from drift, concentrations would be smaller. Similarly, soil concentrations were calculated without factoring in interception of the plant canopy which may reduce deposition on soil to less than 50% if plants are mature and the canopy is closed. For the environmental hazard assessment, toxicity values for the most sensitive

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organism were used. This organism may not be present in Colombia but, as is the case with all hazard and risk assessments, these organisms are surrogates for those that may be present and that have not been tested for sensitivity. In assessing human health hazards, the reference dose was used. This reference dose is based on daily exposure to the chemical for a lifetime and is somewhat conservative for assessing risks from single and infrequent exposures.

For these reasons, it was not possible to estimate risks with any certainty and was the reason for the use of HQs. Although the HQ is not an accurate indicator or predictor of risk from a substance, they may be compared on the basis of the relative HQs. In all cases, these substances presented greater hazards to humans and the environment than glyphosate, whether this herbicide is used in spray eradication or in the production of coca and/or poppy.

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## General conclusions

In general, many of the substances used in cocaine and heroin production and refining are potentially hazardous to human and environmental health. Comparatively, several of the short-listed pesticides are considerably more toxic to humans and nontarget organisms in the environment than glyphosate (plus Cosmo-Flux®). Most of the more hazardous pesticides were found to be insecticides, which are toxic to mammals and other wildlife, as well as to insects. With the exception of endosulfan, these chemicals are registered in Colombia for use in agriculture and their inclusion in this chapter does not imply that they should be further restricted or banned. However, if used improperly, such as in the production of coca and heroin, these compounds have the potential to present significant hazards to human and environmental health, much more so than the hazards identified for glyphosate as used in the eradication of illicit crops.

## Acknowledgements

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## Chapter 7

# Geographical Extrapolation of Pesticide Environmental Fate Data: Challenges, Risks, and Opportunities

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Current knowledge on environmental fate of pesticides is drawn predominantly from Europe and North America. In the absence of local data the resource managers and regulatory agencies of many countries, particularly of developing countries in the Asia-Pacific, have to rely on databases from elswhere. However, behavior of pesticides in the terrestrial ecosystems of these countries can be different due to the inherently diverse chemical properties of soils. Combinations of the chemical properties as well as site-specific conditions determine the fate and behavior of pesticides. These conditions vary greatly among different agroecological zones making the direct extrapolation of data between geographical regions very challenging. This article focuses on the effects of soil and environmental conditions, with particular attention to the chemistry of soil organic matter, associated with tropical and temperate regions on sorption and fate behavior of pesticides. Challenges facing the regulatory decision-makers include an emerging need for better extrapolation of pesticide data among different geographical regions.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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Pesticides have played an important role in tripling of world food production during the past three decades. Agriculture is now growing faster in developing countries and it is projected that by 2030 almost three-quarters of world crop production will come from the developing countries (*1*). Reliance on pesticides in these countries is expected to increase. While some of the environmental benefits of modern agriculture are clear, there is an increasing need to consider any adverse impact of the modern technology on the environment. Availability of cheap, out-of-patent pesticides may present yet another environmental challenge in terms of the impurities associated with the formulations available in the developing countries. The growing public concern about potential adverse impacts of pesticides on ecosystem and human health is fuelling the need for reliable quantitative information on the environmental fate and behavior of pesticides.

For risk assessment of pesticides, the environmental fate data is a crucial input. A substantial body of literature on pesticide fate and behavior is available but mostly from developed countries of Europe and North America. The environmental fate of pesticides in the developing countries, especially in the tropics, remains poorly understood (*2*). Moreover, due to large number of chemicals and site conditions involved, local data are often missing even in developed countries such as Australia and Japan. Consequently, the resource managers and regulatory agencies of many countries have to rely on overseas data without knowing their relevance to the local soil and environmental conditions. However, extrapolation of data between various geographical regions is fraught with a high degree of uncertainty and need to be applied with caution.

Indeed the combinations of chemical properties as well as site-specific soil and environmental conditions, which vary greatly with geographical locations, determine the fate and behavior of a pesticide. Soil properties such as soil pH, the amount and nature of clay minerals, sesquioxides, and soil organic matter (SOM) can be diverse in soils from different climatic regions. For example, many soils of semi-arid tropical countries (e.g. many parts of India, Pakistan, China) are alkaline (pH >8.0), whereas highly weathered soils of the tropics (e.g. Brazil, Malaysia, Australia) and those in high rainfall areas are generally acidic in nature (pH 5.5–6.5). Many soils of the arid zone and tropical countries are low in the SOM content, whereas the volcanic soils, such as in New Zealand and Japan, are rich in allophane clay and contain much higher organic matter contents (averaging 10–12%). Several soils of Australia contain considerable amounts of charcoal (*3*). Obviously many soils in the developing countries in the tropics and in the "Pacific Rim" are very different from the soils of Europe and North America, on which the pesticide databases have originated from (*4, 5*).

In this chapter we assess variations in the chemical nature and properties in soil and environmental conditions among different geographic regions, and their potential influence on pesticide behavior in soil. The focus of this chapter is on the effects of soil and environmental conditions associated with tropical and temperate regions on the sorption and degradation of pesticides. The article concludes by looking to the future trends and challenges in the development and demand for pesticide data extrapolation among different climatic regions.

## Pesticide Fate in Relation to Soil and Environmental Conditions

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Several processes including sorption and interaction with soil particles, degradation, leaching, and volatilization govern the fate and behavior of pesticides in soil and vadose zone. The extent to which these mechanisms control the overall fate of a pesticide is dependent on the properties of the pesticide itself, soil characteristics, climatic factors and management practices, all of which make interpretation of dissipation dynamics difficult.

The nature and properties of soils are driven by a number of soil-forming factors such as parent material, climate, relief, vegetation, and time (*6*). Due to large variations in these factors between temperate and tropical regions, many soils in these regions display correspondingly wide variations in their properties. For example, Australian, Brazilian and Indian Oxisols are highly weathered, with goethite, gibbsite, hematite and 1:1 clays such as kaolinite, low organic matter contents, low pH, and variable charge characteristics. These soils are subject to relatively high year-round temperatures and occasional high rainfalls (*7*).

### Pesticide Degradation and Persistence in Soils

Pesticide degradation in soil occurs via a number of abiotic and biological processes such as oxidation, reduction, hydrolysis, photolysis and mineralization. Pesticide biodegradation in soil has been extensively reviewed by Aislabie and Lloyd-Jones (*8*). Abiotic pathways have been discussed in detail by Wolfe *et al.* (*9*). Some of the differences in the land use, soils and environmental conditions among major climatic regions in relation to their impact on pesticide fate and behavior are discussed here.

#### *Effect of Land Use Associated Conditions*

Land clearing and cultivation induce changes in soil properties. A vast area of Brazil under tropical forest and grassland (natural vegetation) are deforested and cleared every year for crop production (*10*). As in other intertropical regions, deforestation followed by intensive cultivation leads to chemical and biological deterioration of soil properties in Oxisols. Soil fertility diagnostic results found by Sanchez *et al.* (*11*) and Cerri *et al.* (*12*) in Peru and Brazil confirm these propensities, which have often been observed in African Oxisols (*13*).

The most outstanding feature of tropical Monsoon dominated Asia (East Asia, South East Asia, and South Asia) is the intensive use of the land for food production, especially paddy rice cultivation (~90% of the world's rice lands) in its agricultural systems (*14*). Rice is the staple diet for almost half of the world's population and is cultivated in both temperate (Japan) and tropical or subtropical (India) conditions. The soils of this "rice basket of the world" (rice lands) occur mostly on alluvial landforms of Holocene and upper Pleistocene periods, such as floodplains, deltas, and lower terraces. Therefore, many of these soils are not well developed morphologically, and the majority are classified as Entisols or Inceptisols. The biogeochemistry of rice land is controlled by flooding and resulting status and pattern of oxidation and reduction reactions. The inefficiency of decomposition of SOM under submergence also contributes to its accumulation in these soils.

Flooded soils under paddy rice cultivation serve as an effective medium for degradation (both anaerobically and aerobically mediated) of a variety of pesticides used in rice culture. The dynamic aerobic-anaerobic interface of flooded rice soil supports simultaneous occurrence of both oxidation and reduction reactions (*15*). Diurnal fluctuations of pH (6.5–9.5) can facilitate hydrolytic transformation and degradation of rice pesticides. Indeed, it is now well established that many, if not all, pesticides are relatively less persistent in wetland rice soils than in upland soils (*16*). While the chemistry of SOM in rice soils has not been fully investigated, recent work by Oliver *et al.* (*17*) on soils from tropical and temperate regions has shown that land use differences within an agroecological region can have significant influence on the pesticide behavior.

### *Effect of Environmental Conditions*

Environmental conditions, such as temperature, solar radiation, wetting and drying cycles, rainfall intensity, its timing and consequent leaching has long been shown to have major implication on pesticide persistence. Tropical conditions characterized by high temperature and moisture have not only direct effect on pesticide biodegradation but also significantly influence processes such as volatilization, which can be a dominant factor governing pesticide persistence (*18*). Solar radiation contributes appreciably towards photolysis of pesticides present on the soil surface (*19*). Limited studies are reported on pesticide degradation in the tropical soils. Most of them were conducted under controlled

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laboratory conditions whilst comparative data between laboratory and field dissipation in the tropical soil environment are still lacking. The laboratory degradation rates can differ significantly from those obtained from field studies because of the variations in the environmental factors. Laabs et al (*20*), while studying dissipation of soybean and corn pesticides (alachlor, chlorpyrifos, deltamethrin, endosulfan, monocrotofos, simazine, and trifluralin), found that persistence of most of these pesticides in tropical soils under field conditions were lower compared with their persistence under laboratory conditions. Half-life ($DT_{50}$) values of most of these compounds measured in the soil under tropical field conditions were shorter by factors of 5–50 times those under temperate field conditions. Under tropical field conditions the volatilization losses are likely to contribute significantly for the dissipation of pesticides relative to the temperate environment (*18*). These losses may also be substantially large in the field; even pesticides such as metolachlor rated as non-volatile, can suffer substantial volatilization losses (>20% of the applied amount) (*21*).

Comparing pesticide persistence data from tropical and temperate regions Racke (*2*) reported that the persistence under tropical condition was significantly shorter than that under temperate conditions and concluded that soil characteristic variations were less important than environmental conditions for pesticide degradation. However, variations in soil properties such as soil pH, type of organic matter and mineral matter, microbial population types and densities within a given or among various geographical regions have clearly a major influence on pesticide behavior, as briefly discussed in the proceeding section.

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### *Effect of Soil Properties*

A range of soil properties, such as soil pH, its mineral and organic carbon content, its water content, temperature and microbial status are also important determinants of the persistence of pesticides. Soil pH influences greatly the hydrolytic processes of pesticide dissipation and depends on the nature of the chemical e.g. some compounds are acid hydrolyzed and others base hydrolyzed. In New Zealand >99% agricultural soils are acidic in nature. In contrast, a large area of soils in Australia, India, Pakistan, and other semiarid tropics are alkaline. Sarmah *et al.* (*22*) demonstrated the increased persistence in sufonylurea herbicides with increasing soil pH in different soils (Figure 1). In New Zealand, therefore, soil pH is a dominant factor for hydrolytic reactions particularly for sulfonylureas and other ionizable pesticides. A contrasting behavior is observed in alkaline soils. Abioitc (catalytic) reactions are dependent on the properties of soils and the climatic factors particularly temperature. Therefore, hydrolytic degradation of pesticides in temperate soils with lower average year-round temperatures is likely to be lower than in the soils of tropical regions.

Both clay mineral and humic substances can produce oxygen-reactive species (singlet and atomic oxygen, and hydroxyl radicals) when exposed to sunlight (*19*) and contribute towards enhanced photodegradation. The type of dominant clay mineral present in soil may also catalyze the oxidation reactions (*23*). In field studies on an allophanic soil of New Zealand, higher degradation rates of the herbicide clopyralid were found in both the bare ground and pasture unshaded plots compared to shaded plots (*24*). Furthermore, microbial populations may be different in tropical *vs* temperate soils. Monkiedje *et al.* (*25*) studied degradation and persistence of metalaxyl in tropical (Cameroon) and temperate (Germany) soils and found different degradation rates in both soils and suggested that different microbial populations had different degradation preferences. Sparling *et al.* (*26*) demonstrated that mineralization of atrazine was significantly affected by the soil temperature, water content, and the atrazine-degrading microbial population present in the soil under New Zealand soil environmental conditions.

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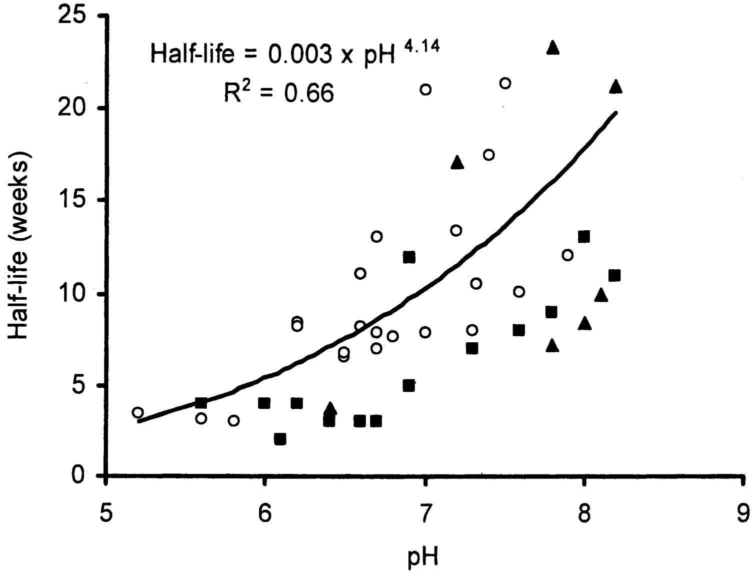

*Figure 1. Degradation of chlorsulfuron as influenced by soil pH. Different symbols represent different datasets. (Reproduced with permission from reference 22. Copyright 1998.)*

## Pesticide Sorption Behavior

Sorption is one of the major processes affecting the fate of pesticides in the soil environment through its effect on bioavailability, biotic and abiotic degradation, and transport over land or through the soil profile. While pesticide sorption properties have been extensively studied for temperate soils and climates (reviewed by *27, 28*), such studies in tropical soils have been limited to relatively few compounds.

For non-ionic pesticides, organic matter is the most important sorbent in soils and strong correlation has been frequently observed between sorption of non-ionic pesticides and SOM content. Organic matter can be broadly classified into humic and non-humic, either of which can play a role in pesticide sorption. Humified material is often a stronger sorbent for non-ionic pesticides because of the presence of oxygen-containing functional groups such as –COOH, phenolic, aliphatic, enolic, –OH, and C=O, but this is not so for ionic pesticides due to a range of possible sorption mechanisms (*29*). From the standpoint of interactions of non-ionic pesticides with SOM, significant differences in the chemistry of organic matter in soils from different geographical regions are expected. An overview on sorption of non-ionic pesticides, with particular reference to the nature of SOM, is presented in the following section.

### *Variations in Sorption of Pesticides Observed in Soils of Different Countries*

Sorption of non-ionic compounds to soil and sediment in aqueous systems has so far been largely attributed to partitioning of the compound between water and the organic matter (*30*). The sorption coefficient or partitioning coefficient ($K_p$) of a pesticide is therefore expressed per unit mass of organic carbon in soil, such that: $K_{oc} = K_p/f_{oc}$, where $f_{oc}$ is the fraction of soil organic carbon. It is commonly assumed that $K_{oc}$ is approximately constant for a given non-ionic compound and that $K_p$ of a compound in any soil or sediment can be estimated from $K_{oc}$. The temptation to regard $K_{oc}$ as a constant has been universal and the relationship has become the basis of assessment of pesticide fate and movement in soil. During the past decade, the mechanism of sorption to SOM has received further attention, and a substantial body of evidence has been presented over the years that has established that the sorption mechanisms in soils involve more than just simple partitioning (*31*).

A large variation in $K_{oc}$ among soils has been recently reported. For example, Ahmad *et al.* (*32*) investigated sorption of carbaryl and phosalone in 48 soils from tropical and temperate soils of Australia, Pakistan, and the UK. They noted that regression of $K_p$ for the pesticide carbaryl and phosalone against the total organic carbon content of the soils could account for only 53 and 46% of

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the variance in $K_p$, respectively. The sorption data for carbaryl in soils of various countries have been depicted in Figure 2. The $K_{oc}$ values decreased in the order of Pakistani > Australian > New Zealand > UK soils with a variation of 17-, 11-, 6- and 2-fold, respectively. The mean values of $K_{oc}$ in tropical/subtropical soils of Pakistan and Australia were larger than the corresponding values from the temperate soils of Europe and North America. The soils from the temperate regions of UK and New Zealand generally are richer in organic matter. By contrast, the more humified SOM in the warmer climates of Australia and Pakistan was responsible for the higher $K_{oc}$ in these soils.

Other differences, such as land use and type of vegetation associated with the SOM in the soils, might have also contributed to the variations in sorption. Sorption of pesticides can also vary between soils of the same region because of the different affinities of SOM for pesticides and interplay of other soil properties. For example, Gerritse *et al.* (*33*) reported higher $K_{oc}$ values of atrazine by an order of magnitude in five sandy soils from Western Australia compared with the values reported for soils from other regions of Australia.

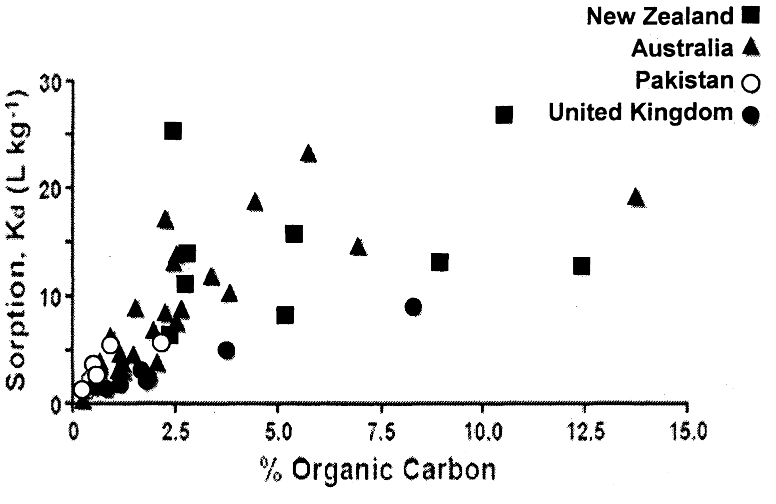

*Figure 2. Variations in sorption coefficients for carbaryl insecticide, in relation to organic carbon in soils of various countries.*

Land use variations can also influence pesticide sorption. For example, sorption in soils under native vegetation was found to be significantly different from that in the soils under market garden (*34*). Some more data reflecting variability in $K_{oc}$ values for selected non-ionic compounds in soils are presented in Table I.

**Table I. Ranges of Variations in Sorption Coefficient ($K_{oc}$) Values of Nonionic Compounds in Various Soils**

| *Compound* | *$K_{oc}$ (L $kg^{-1}$)* | *Organic Carbon (g $kg^{-1}$)* |
|---|---|---|
| Carbaryl | 64–4318 | 3–138 |
| Chlorpyrifos | 2164–35170 | 21–114 |
| DDT | 131541–443137 | 28–67 |
| Dibenzanthracene | 566334–3066996 | 14–71 |
| Disulfoton | 810–8723 | 3–79 |
| EDB | 36–160 | 9–374 |
| Lindane | 736–2589 | 21–353 |
| 3-Methycholanthrene | 363764–6396040 | 14–71 |
| Napropamide | 190–2108 | 2–41 |
| Parathion | 314–15861 | 3–105 |
| Piperophos | 124–13149 | 31–176 |
| Phorate | 364–6862 | 3–547 |
| Phosalone | 1506–85528 | 3–138 |

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The SOM can vary from soil to soil in its elemental composition, polarity, aromaticity, condensation, and degree of diagenetic evolution from a loose polymer to condensed coal-like structures (*36*). Therefore, age and type of SOM can affect sorption of non-ionic pesticides. However, the $K_{oc}$ does not take into consideration these properties, which can affect sorption of these compounds.

### *Geographical Variations in Organic Carbon Contents and Chemistry*

Compared with temperate zones, tropical temperature regimes are largely warmer and exhibit much less variation from season-to-season. In tropical regions, the mean daily incident solar radiation is about twice that of temperate regions. Since the formation and decomposition of SOM is primarily microbially mediated, the temperature at which these processes occur is likely to control the rates of organic matter transformation, provided that other soil conditions such as oxygen, soil water content, clay type and content, and pH are similar (*37*). Thus the decomposition rate of organic matter in the agricultural soils in tropical regions is likely to be considerably faster (up to 5 times) than in the soils of temperate regions (*38*). Consequently, the soils of the tropical regions contain relatively lower organic matter contents than those from the temperate regions. In tropics, decomposition is particularly sensitive to the soil moisture regime and the type of land cover. Generally, cool climates (as those of temperate regions)

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promote accumulation of SOM, whereas humid and warmer conditions, as occur in tropical and subtropical regions, favour mineralization of organic matter.

The chemical composition of SOM is determined by site-specific environmental conditions. Zech *et al.* (*39*) investigated eight soil profiles from temperate, subtropical, and tropical regions. Correlating chemistry of the SOM with site factors, they found that temperature and the temperature/precipitation ratio influenced mainly the aromatic component of the bulk soil samples. In soil horizons with low C:N ratios and high pH, aromaticity tended to be somewhat higher. This shows that advanced stages of humification are characterized by higher aromatic components of the SOM. About 73% of aromaticity could be elucidated by the variation of temperature/precipitation ratio and the remaining by the variation of soil pH and the C:N ratio (*39*). Studying the humic acids of five Kenyan soils, Arshad and Schnitzer (*40*) also found a positive relationship between the temperature/precipitation ratio and aromaticity. More recently, the effect of local climate on the chemical composition of SOM has also been pointed out by Dignac *et al.* (*41*) in a study with North European soils.

In West African savannas, which make up the bulk of the tropical land area, and also in the tropical zones of Australia, the decomposition process can be accelerated by fire (common in the regions) and herbivory. Both of these processes are highly influenced by land use decisions. Indeed many soils of Australia contain a significant proportion of charcoal, which may represent as much as 50% of the soil carbon (*42*). In many other tropical and sub-tropical countries (e.g. India and Pakistan) organic inputs into soils encompass a wide range of materials including crop residues, green manures, prunings, and animal, household and agro-industrial wastes. Such materials have varying compositions, and proportions of organic matter depending on the handling and processing.

### *Variations in the Chemical Composition of Soil Organic Matter*

Due to difficulties in isolating unaltered SOM and characterizing its properties, the mechanisms involved in the sorption of organic compounds are not clearly understood. Also methods of extraction of organic materials from soil may alter its nature, and the observed properties may differ from those of organic matter *in situ*. However, the development of solid-state $^{13}C$ NMR spectroscopy has provided a useful tool for the examination of SOM by using whole soil samples as well as fractionated ones.

Krosshavn *et al.* (*43*) investigated soil samples from different vegetational backgrounds using soild-state $^{13}C$ NMR and found that both the vegetation source and degree of humification substantially influenced chemical structure of the organic matter in those soils. The aromatic fraction of the SOM ranged from 8.5 to 13.7%. The aromatic carbon increases with increasing decomposition

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(*44*). Fingerprinting of soils through $^{13}$C NMR from different regions of Australia and Pakistan revealed clear differences in the chemical composition of organic matter (*45*). In the 25 agricultural soils from Pakistan and Australia, the O-alkyl C varied from 34 to 46%, alkyl C from 17 to 38%, and the aromatic C (aryl + O-aryl C) ranged from 10 to 31% (Figure 3).

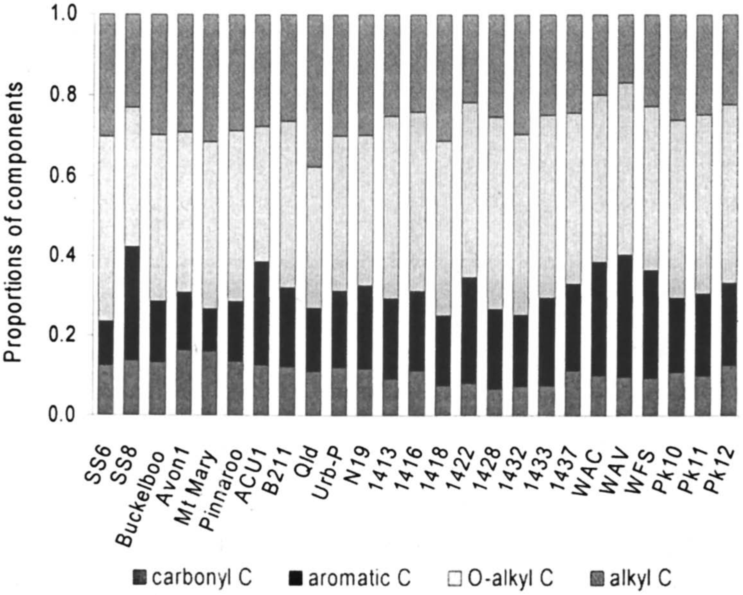

*Figure 3. Variations in the structural components of organic matter in some soils of Pakistan & Australia (Adapted from ref 45 American Chemical Society)*

It has been difficult to determine the molecular composition of SOM. However, recently Ahmad *et al.* (*46*) were able to estimate the molecular components of SOM in a range of soils from various agro-climatic regions of Australia and Pakistan, which revealed substantial variations in the molecular nature of SOM (Figure 4). Australian sandy soils contained the largest proportions of lignin or lignin-derived materials followed by forest soils. A number of Australian soils contained appreciable amounts (>10%) of charcoal. Some soils from Pakistan contained much higher proportion of lignin. Arai *et al.* (*47*) also characterized the nature and composition of organic components in two soils (an Alfisol and a Vertisol) from semi-arid tropics of India and found fulvic acids to be major constituents: about 90% in the Alfisol and 70% in Vertisol, suggesting a faster decomposition of SOM in Alfisol.

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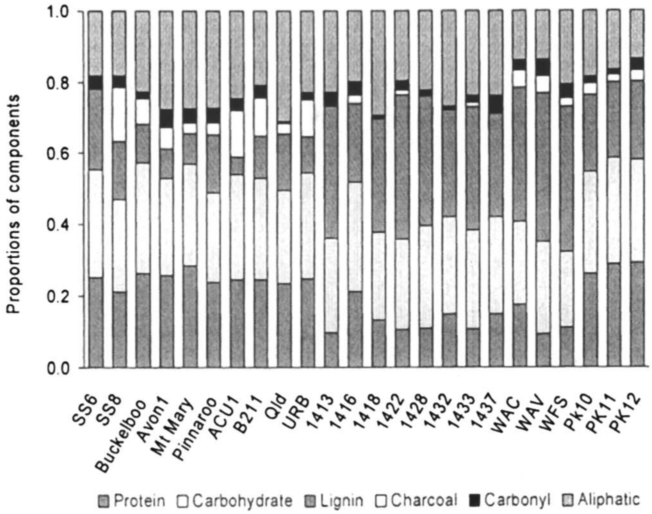

*Figure 4. Variations in the molecular components of organic matter in soils of Pakistan and Australia (Reproduced with permission from reference 46. Copyright 2006 BlackwellPublishing.)*

### *Relationships of Soil Organic Carbon Chemistry with Pesticide Sorption*

While it is useful to relate the chemical nature of SOM to the sorption for hydrophobic organic compounds, little is known about the correlation between the chemical and physical properties of SOM and the behavior of pesticides in soils. The few available studies on this topic have been restricted to sorption of organic pollutants on commercial and pure humic materials (*48–49*), even though they are not good representative of natural humic substances. However, Ahmad *et al.* (*45*) investigated relationships between the $K_{oc}$ values of carbaryl and phosalone and the various structural components of SOM in a range of soils from various agro-ecological regions. They found that the aromatic component of the SOM had a significant impact on sorption of the two pesticides. A strong positive exponential correlation of $K_{oc}$ values with aromaticity ($r^2 = 0.94$ and 0.95 for carbaryl and phosalone, respectively) was observed, which indicated that aromaticity of the SOM was a key structural parameter that regulates sorption of non-ionic pesticides.

Kleineidam *et al.* (*50*) investigated sorption of phenanthrene in sorbents derived from sedimentary rocks containing organic materials of different origin and maturity, including those from marine and terrestrial plant debris, lignite, coal, and charcoal and found that the $K_{oc}$ values for these materials varied by three orders of magnitude (Figure 5). Exceptional high $K_{oc}$ values were noticed for charcoal and coal, and the sorption isotherms for these materials were highly nonlinear. The molecular nature of SOM as determined from the NMR spectra and the elemental composition, and the multiple regression analyses showed that among various molecular components, relationships using both lignin and charcoal contents were highly correlated with the $K_{oc}$ values of carbaryl and phosalone (*46*). Lignin has also been reported to show high binding propensities for other pollutants (*51*). From these results it is very likely that pesticide retention in burned-over fields and those containing wind-blown charred carbon particles can be high. This demonstrates that extrapolation of data may hold well for groups of closely related soils in a region, but as the structural and chemical similarity of the SOM diminishes, so does the validity of extrapolating data.

### *Other Soil Properties Affecting Pesticide Sorption*

Weathered soils with variable-charge minerals constitute an important group of soils in tropical and sub tropical agricultural regions, such as Australia, South and East Asia, and Central Africa, and contain substantial proportion of amorphous or crystalline oxides and hydrous oxides, which have pH-dependent charge characteristics (*52*).

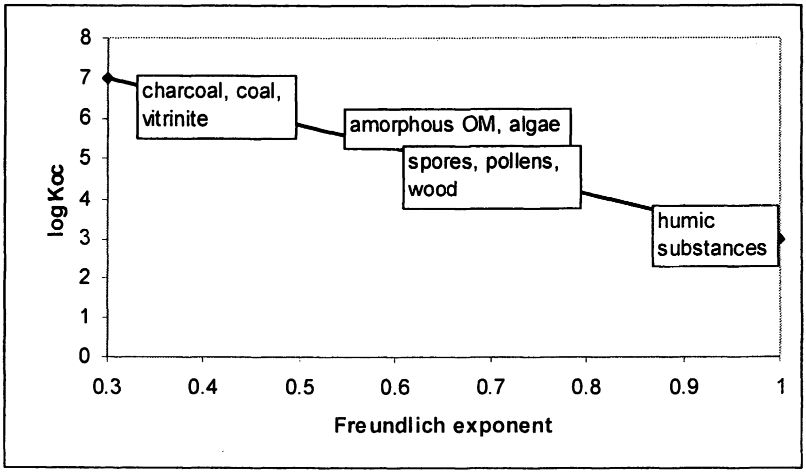

*Figure 5. Sorption coefficients and the isotherm non-linearity indicated by Freundlich exponent values for various types of organic materials (Source: Reproduced from reference 50 American Chemical Society.)*

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An estimated 60% of the soils in tropics have these variable-charge minerals compared with only 10% of the soils belonging to the temperate regions (*53*). Such soils display significant anion exchange capacities. The large humus accumulation in allophanic soils (many New Zealand and Japan soils) containing large amounts of amorphous or crypto-crystalised minerals is well known. This is mainly attributed to very stable humus-Al, Fe- complexes, which may likely be protected from bacteria and enzymes in micro aggregates rather than by a specific effect of allophane and associated minerals (*54*). It is likely that SOM in allophanic soils is highly decomposed; it may be rich in carboxylic and aromatic carbon, giving them very high affinity to Al/Fe-oxides/hydroxides (*55*). Clay minerals can make a significant contribution to pesticide sorption, particularly to ionizable pesticides. Weber (*56*) reported that s-triazine herbicides were readily sorbed onto various clay minerals including illite, montmorillonite, and kaolinite. The clays in highly weathered soils of the tropical regions are dominated by kaolinite (1:1 type of clay minerals possessing low surface area and low negative charge) and have a lower capacity for pesticide sorption than 2:1 type clay minerals like montmorillonite and vermiculite (*57*). Oxisols, which are rich in non-crystalline to poorly crystalline Al and Fe oxides, have been shown to significantly sorb anionic pesticides, as these can carry net positive charge at ambient pH (*58*). In contrast, cationic pesticides (e.g. paraquat, diquat) show relatively lower sorption in such soils because of the lower cation exchange capacities of the soils despite their high clay contents.

Soil pH is another factor that influences the sorption of pesticides. It regulates the ionization state of weakly acidic and weakly basic compounds and the ionizable functional groups present on various soil constituents. Basic pesticide molecules become protonated at lower pH and, therefore, more strongly sorbed to the soil colloids. Conversely, the acidic pesticides ionize and become anions as pH increases (one or more pH units above the $pK_a$) and thus sorption is less pronounced (*22*). Figure 6 shows the effect of pH on the sorption of chlosulfuron in soil. Rocha *et al.* (*59*) also reported that imazaquin sorption in Brazilian soils increased with decrease in soil pH. However, when the net electric potential was positive and the organic carbon content of the soil was low, it was not possible to predict imazaquin sorption considering just molecule speciation and its partition to the organic domain of the soil. In these soils Fe and Al oxides play a major role in the overall sorption, since they dictate the net electrical charge, as well as the electric potential of the soil. Therefore, it becomes difficult to extrapolate data to be applicable for such types of soils owing to the complexity of the interactions among ionizable compounds and highly weathered soils since different soil attributes and sorption mechanisms act simultaneously.

In the preceding sections various factors affecting the behavior of pesticides in soils of various countries have been considered. A detailed account of pesticide behavior in soils would involve many other parameters. Nevertheless,

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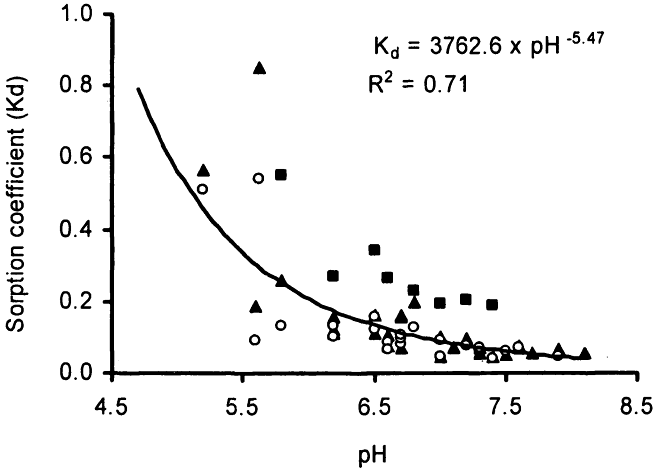

*Figure 6. Effect of soil pH on sorption of chlorsulfuron. Different symbols represent different data sets (Reproduced with permission from reference 22. Copyright 1998.)*

the extent of sorption by soil constituents in general, and the SOM in particular, is expected to be a primary factor influencing pesticide behavior. Many soils in tropical regions, containing an appreciable amount of variable-charged components and higher pH, have properties that are striking in contrast to those of the soils in temperate regions. These factors also need to be taken into account whenever pesticide data are extrapolated between different climatic regions.

## Inadequate Use of Concepts Implemented in the Extrapolation of Pesticide Data

Major sources of error in the extrapolation of pesticide data is the use of concepts that do not apply to a particular situation e.g. inadequate use of the $K_{oc}$ approach and the parameterization of pesticide dissipation. It is generally considered that $K_{oc}$ is normally distributed and therefore $K_d$ is obtained by multiplying $K_{oc}$ by fixed values of soil organic carbon (*60*). In other cases $K_d$ values are derived on the basis of fixed values of $K_{oc}$ and distribution of soil organic carbon values, mostly from published databases (*61, 62*).

Reliance on $K_{oc}$ implicitly assumes that SOM is the sole sorptive phase and ignores the contribution of other soil properties such as type and amount of clay, CEC, pH, and the nature of SOM itself. In fact the $K_{oc}$ approach was introduced to reflect the linear relationship between the $K_d$ and organic carbon content and

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was initially established for hydrophobic non-ionizable compounds (*63*) for the reason that this variable has been considered independent of any particular soil. Even so, the $K_{oc}$ concept tends to be used for all classes of chemicals (including herbicides) regardless of their ionization status. But, the $K_{oc}$ approach is often invalid for ionizable compounds, since there is often no sole and clear influence of organic carbon content on sorption of these compounds. Contribution and subtle interplay of several additional factors such as amount and type of clay, pH, and CEC may exacerbate the difficulties in extrapolation of sorption coefficient values of such compounds. Thus knowledge on various soil properties that influence herbicide sorption in soil is critical to identify soil-herbicide combination with varying degrees of risk for groundwater contamination.

Another problem that plagues predictions or extrapolation methods is the approach generally used in the estimation of $K_{oc}$ from empirical equations (e.g. *64, 65*). This approach is far preferable to complete reliance on empirical correlation which precludes extrapolation out of the narrow range of substances or the system properties upon it is based. The approaches stated above are likely to have considerable effects on the results because of the unrepresentative input values used under a particular situation.

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## Future Directions for Better Extrapolation of Pesticide Data

Extrapolation of pesticide data is likely to remain the main tool for risk assessment of pesticides for the foreseeable future. Therefore more appropriate ways of data extrapolation need to be developed to account for the inadequate use of $K_{oc}$. For ionizable compounds there is a need to consider other input properties such as pH, clay type, CEC, and combining the chemistry of soil organic carbon, accurately to rank and improve predictions of the risk posed by these compounds. In particular for acidic pesticides it is crucial to consider simultaneously the physical and chemical reactions of the ionized and neutral species as well as changes in the electrostatic properties of the soil surface with changes in parameters such as pH, ionic strength, and ionic composition.

For the next several decades, land use and land cover changes will continue to be the most significant and obvious aspects of environmental changes particularly in the tropics. Therefore the effects of atmospheric composition and climatic change may become paramount with respect to the environmental fate of pesticides and other xenobiotics. The types and magnitudes of future state of driving variables in the context of pesticide fate and effects are not yet sufficiently known to make reliable ways for extrapolation of pesticide data among various geographical regions. The development of predictive approaches has already started advancing, but must continue and accelerate with emphasis on extrapolation to tropical soils.

Recent work relating the chemistry of SOM with pesticide sorption has started to reveal some interesting observations, such as the relationship of $K_{oc}$ with aromaticity of organic carbon (*45*) and with the molecular components of organic carbon such as lignin and charcoal contents (*46*). While this may not hold good for all pesticides, the approach is showing some promise and warrants further attention. The next challenge, of course, would be to establish if the $K_{oc}$ concept can be refined taking into account the chemical nature of organic carbon. Furthermore, simpler tools would need to be developed to characterize the chemical nature of SOM as not many laboratories in developing countries can at present afford access to solid-state NMR.

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## Conclusion

Unquestionably, pesticides will continue to play a vital role in global agriculture. Given that a large number of chemicals in use with the many variations among soil and climatic conditions that exist among geographical regions, it will simply not be practicable to obtain adequate site-specific pesticide data. Therefore, there is an urgent need to develop tools that can help customize data from one region to another and thereby allow a wiser use of available environmental fate data. This warrants greater understanding of sorption and fate behavior of pesticides in soils from tropical and temperate regions. Clearly, the current level of understanding the role of the chemistry of SOM in regulating pesticide sorption, fate, and effects in soils of different climatic regions is inadequate. As the behavior of the vast majority of pesticides and other xenobiotics is strongly dependent on the chemical nature of SOM, we must continue to probe links between the pesticide behavior in soil environment and various structural and molecular components of SOM complex. There is not only need for better tools for effective extrapolation of data among regions but also the recent research, showing considerable promise, need to be pursued with vigour to refine existing concepts.

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## Chapter 8

# Rain Simulation to Estimate Pesticide Transport in Runoff

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Rainfall simulator studies are useful in understanding and managing pesticide transport in runoff water, having advantages in studying contaminants such as labile pesticides. They allow timely comparisons of various factors affecting pesticide transport, which is difficult using studies of natural rainfall in catchments, and facilitate direct comparison of various pesticides with contrasting properties. Rainfall simulation complements, rather than replaces, field studies under natural rainfall. An experimental framework, which relates pesticide concentration in runoff to that in the soil surface and thus considers application rate and prior dissipation, is used to compare data from rainfall simulator plots and natural rainfall catchments. Simulator studies can also provide time-variable partition coefficients relevant to runoff generated under rainfall and data for pesticide washoff from plant canopies and crop residues.

Quantification of pesticide transport in surface runoff is one of the weakest areas of our understanding of environmental fate and behavior of pesticides (*1*). The literature on pesticide runoff presents a seemingly random collection of runoff concentrations. These data vary with multiple factors, such as the sites hydrology and erosion, the pesticide properties (e.g. partitioning between water

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

and solids, propensity to leaching), application method, and in particular, the application and dissipation rates and timing of the event(s) after spraying. Most studies involve only a small number of pesticides, so it is uncertain if the responses observed result from the pesticide used or the conditions of the study.

A better index of pesticide runoff would allow comparisons of the behavior of new products being registered or alternative products intended to improve water quality. There are many different pesticides, with a wide variety of chemical properties (*1*). Hornsby *et al.* (*1*) list properties of 343 pesticide active ingredients; many more compounds, metabolites and potential contaminants exist. The properties of these chemicals can impact on a variety of processes involved between application and runoff arriving at the edge of a field. An important issue for their management and regulation is how they behave relative to each other as a result of their different properties.

Measurement of pesticide runoff from agricultural fields and plots indicate differences in concentrations and total losses between various compounds (*2,3*). However, these studies do not always separate out (quantitatively) the contribution of various causative factors behind these differences. We suggest a framework for distinguishing the main factors that determine pesticide transport in runoff. One possible cause of differences is the use of rainfall simulators as opposed to catchments under natural rainfall (*2*). In this paper, we discuss the use of rainfall simulators to collect data on pesticide runoff and washoff from plants, and use the framework to compare data from simulators and catchments.

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## Pesticide Runoff Processes

Difficulties in characterizing pesticide runoff arises because transport is the outcome of a series of processes, many of which may be affected by the properties of the pesticide and conditions of the site:

- application, dissipation, rainfall by leaching, runoff extraction and runoff transport, acting on several compartments or surfaces (e.g. plant canopy, crop residues, soil),
- transport in several phases of runoff (particulate, water, sediment), and
- partitioning involving soil/sediment-water at several of these stages.

Dissipation may involve losses by volatilization, degradation or transformation (chemical, biological or photochemical) and, for more mobile pesticides, leaching or diffusion deeper into the soil. Dissipation from the surface 10-25 mm is relevant to pesticide runoff (*4*), rather than that in the deeper bulk soil. Because of dissipation, the 'source strength' decreases so the timing of the runoff event after application is critical. Rapidly dissipated pesticides present a shorter period of risk; slow dissipation extends the period of risk. When runoff does occur, pesticide extraction is affected by the hydrologic and erosion characteristics of the event.

Wauchope and Leonard (*5*) found that 'edge-of-field' maximum pesticide concentrations in individual runoff events reported in the literature could be related, to within about one order of magnitude, to application rate, time of the event after application and an 'availability index'. The availability index was grouped into four classes according to formulation (EC, wettable powders, granules), solubility and placement (on foliage, soil surface, or incorporated). Though simplified, the model showed the relative importance of several factors.

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## Pesticide Runoff Framework

Silburn (*6*) used a quantitative framework to compare data from various studies, including rainfall simulators and catchments. To simplify, we will consider pesticide runoff from the soil (rather than plant canopies). The framework, inspired by Leonard *et al.* (*7*), has the following concepts:

- Concept 1: pesticides in runoff are extracted from a shallow soil depth (*4*); for practical sampling purposes a 0-10mm (*7*) or 0-25mm (*6*) layer.
- Concept 2: the concentration of a pesticide in this soil surface layer is a function of application rate, initial loss and dissipation rate (half-life). Concentrations of pesticides can vary over several orders of magnitude after application, resulting in variation in runoff concentrations.
- Concept 3: a large proportion of the variation in pesticide runoff is related to the concentration in the soil surface at the time of the event (*3,6-8*). Pesticide runoff is represented by the flow weighted event mean concentration. The conceptual framework is illustrated in Figure 2 using the data from (*7*) for four herbicides dominantly transported in the water phase. The slope of the linear relationship is defined as the runoff extraction ratio.

Maximum potential concentrations, calculated as the total mass of pesticide in a soil layer divided into the total rainfall (arbitrarily chosen as 50 mm), are also shown. These define an approximate upper limit for pesticide runoff.

They indicate that the pesticide in runoff measured by Leonard *et al.* (*7*) is explained by extraction of about 20 percent of the pesticide in the 0-10 mm soil layer (or complete extraction from 0-2mm) at higher concentrations and somewhat less extraction at lower concentrations. The "other" variation indicated in Figure 2 is related to:

- Errors in measured soil and runoff concentrations, which can be considerable because of spatial variability in soil concentrations and sampling depth, and insufficient samples through the runoff hydrograph.

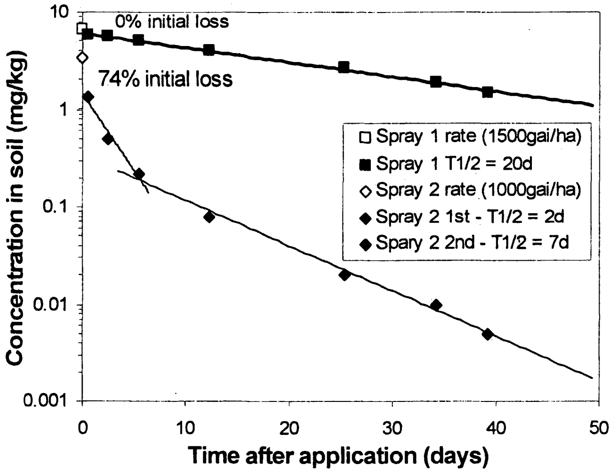

*Figure 1. Concentration in the soil (0-25mm) for two pesticides, 1 – moderate, single phase half-life, 2 – rapid initial loss, rapid multi-phase half-life. (6).*

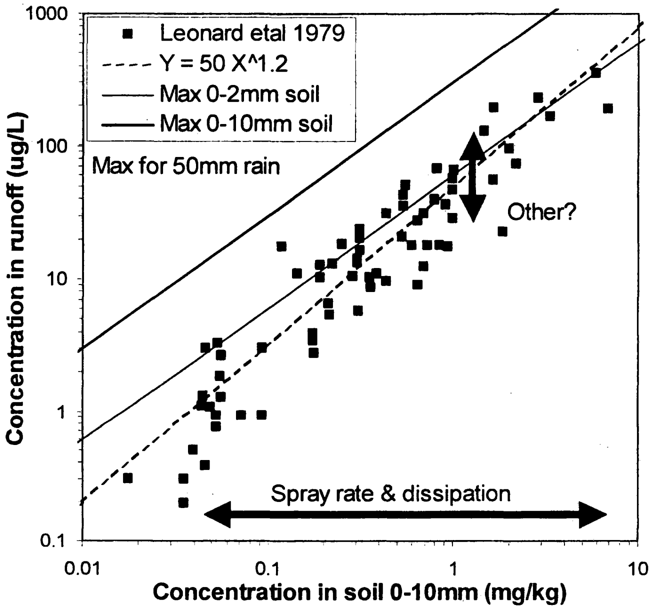

*Figure 2. Conceptual framework for concentration of pesticide in runoff and soil surface, with data from Leonard et al. (7) for herbicides dominantly transported in the water phase.*

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- Different concentrations in the actual runoff mixing zone and the sampled soil depth, due to variation in the shape of the concentration-depth profile, e.g. with time after application and application method.
- Variation between events in:
    - rainfall and runoff rates (e.g. water-phase extraction and sediment movement both increase with rainfall intensity/kinetic energy)
    - infiltration and leaching (which may also vary with partition coefficient)
    - sediment load and sorbed pesticide (varying with partition coefficient)
    - form and depth of erosion (inter-rill, rill) (7)
    - interflow/reemergence of leached pesticide (if low partition coefficient)
    - land cover (crop or stubble) which may or may not have been sprayed.

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The sediment load in runoff will effect transport of pesticides, particularly for high sediment concentrations or for pesticides with higher partition coefficients. Leonard *et al.* (*7*) presented data for paraquat, which is highly sorbed. Runoff transport was related to sediment concentration in runoff (SC) as well as the paraquat concentration in the soil. They summarized the data as:

$$\mu g/L \text{ in runoff} = 1000 x 2.16 x [(mg/kg \text{ in soil}/1000)\ x\ SC\ g/L]^{0.83}\ (R=0.97).$$

This equation is illustrated in Figure 3 for three sediment concentrations, covering the range measured in the study. These data coincide with the data for water transported herbicides over certain ranges of sediment concentration. For instance, with moderate concentrations of sediment in runoff (e.g. 5-10 g/L), runoff concentrations of paraquat are similar to those of the water transported herbicides for soil concentrations from 0.05 to 10 kg/kg.

Silburn (*6*) measured soil and runoff concentrations for 15 pesticides, in three rainfall simulator studies, clay soils in cotton fields in Australia. The data were represented by the relationship: Concentration in runoff (µg/L) = 28 *x* concentration in soil (mg/kg). This relationship ($Y=28.X$) and the equation derived by Leonard *et al.* (*7*) ($Y=50.X^{1.2}$) (Figure 2), are compared with other data in later sections.

## Use of Rainfall Simulators to Study Pesticide Runoff

Rainfall simulators have been used to study infiltration, runoff, soil erosion and water quality for over 50 years. Considerable early work was done to characterize natural rainfall (*9,10*), to obtain simulated rainfall similar to natural rainfall (*11*) and design various mechanisms (*12-16*). Pesticide scientists were reasonably early adopters of rainfall simulators (*17-18*). Rainfall simulators augment rather than replace plot and catchment studies under natural rainfall.

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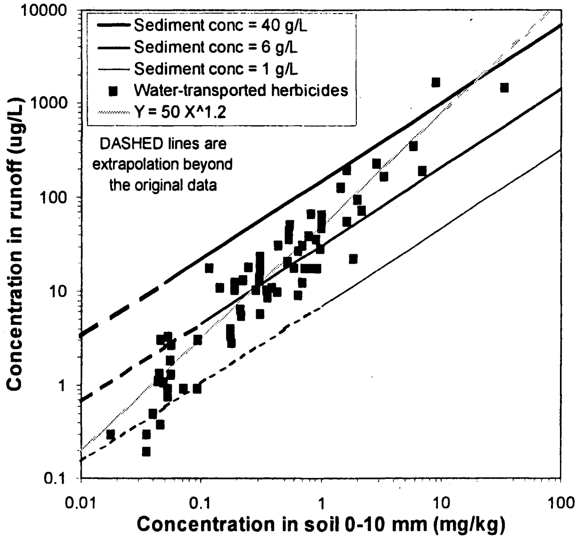

*Figure 3. Conceptual framework for concentration of pesticide in runoff related to concentration in the soil surface, showing the effect of the concentration of sediment in runoff on runoff transport of a highly sorbed pesticide.*

## What are Rainfall Simulators?

Rainfall simulators apply rainfall at a controlled rate to a defined area of land (a plot) or to containers in a laboratory, to allow measurement of infiltration, runoff and contaminants in runoff. Various rainfall simulators are described by (*12-16,19*). Their use and interpretation are reviewed by Renard (*20*), Wauchope *et al.* (*21*) and Connolly *et al.* (*22*). They are designed to produce rainfall with similar characteristics (e.g. drop sizes, energy etc) to natural rain for their intended range of rainfall intensities and geographic region.

The plot area is typically <1m$^2$ to tens of m$^2$. Simulators have been built to rain on larger areas, but involve more work in setting up and a much larger water supply, limiting the number of plots that can be run in a given time. The plot size must be adequate for the physical process of interest. For instance, soil erosion by water is scale dependent as rill erosion occurs on larger plots but not on small plots (*23*). However, if these processes are not relevant smaller plots may be adequate and more convenient. Silburn (*6*) found no difference in runoff of several pesticides from small and large plots on low sloping plots.

The simulator we used in pesticide studies in cotton fields (*2,24-25*) (Figure 4), described by (*19*), is based on the design of Meyer and Harmon (*15*). It uses in-line modules of oscillating flat-fan nozzles spraying downwards, wetting a area 13 m long and 2.5 m wide. A single module was used for short (2m by

1.6m) plots. Rain was applied to two adjoining plots (cotton furrows) simultaneously. Rainfall characteristics are similar to natural rain in eastern Australia. Rainfall intensity is controlled by the frequency of passes of rainfall over the plot using an electronic control box. Rain was applied for 40 min or more, at intensities of 70 to 100 mm/h. Reasonably intense storms were applied, as they cause the majority of total soil loss in this type of environment. It is important that management practices are effective for such storms.

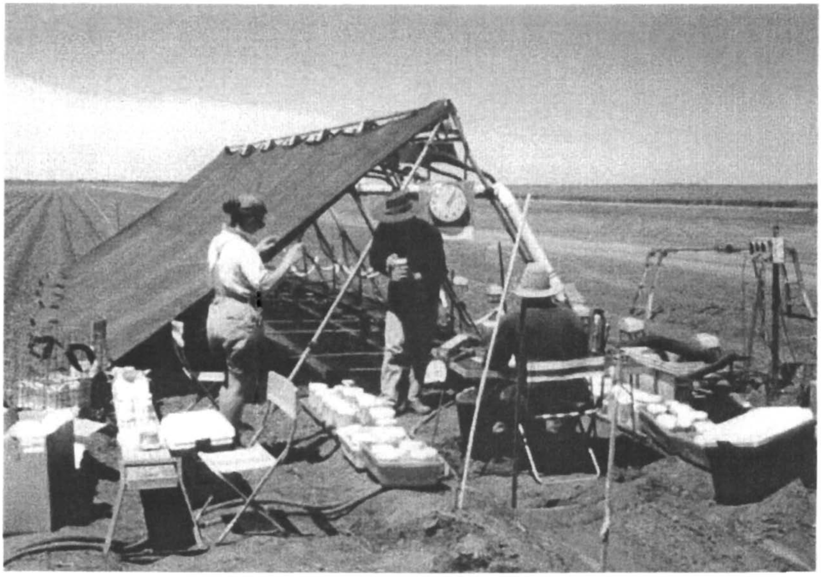

*Figure 4. Rainfall simulator on two cotton furrow plots, with sample bottles laid out for runoff and sediment, sediment size and pesticide runoff (6).*

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We ran two plots per day with the large simulator or four to six plots per day with the small simulator, using four to six people in the field and one or more in a nearby laboratory. A large supply of good quality water must be transported (usually rain water). Automated devices are available to measure the rate of runoff from the plot. However, measurement of fill time and mass of hand-samples of runoff generally suffices. Plots were sprayed at specific times prior to applying rain, with several pesticides, aiming for a range in properties (e.g. sorption and half-life). Concentrations in the soil surface were measured through time. Time since spraying was often included as an experimental variable.

The cost of analyses is a constraint in studying pesticides. Sufficient samples are needed during the runoff event to obtain good estimates of the event average concentration and total loss. Analysis costs may be prohibitive if all runoff samples are filtered and used to determine the pesticide concentrations in water and sediment phase separately – costs are doubled. Yet these data allow an important partition coefficient to be calculated. To minimize the cost, Silburn (*6*) tested the use of a bulked runoff sample to represent the flow weighted concentration and the water and sediment phase pesticide concentrations for the entire event. Samples contained six pesticides with contrasting properties. The bulked samples were taken by adding a portion of runoff to a single bottle at a number of times during the event. Sample must include the entire runoff stream and be of equal duration to achieve flow weighted averaging. Concentrations in bulked samples compared well with flow weighted mean concentrations calculated from samples through the event ($Y = 1.011\ X$, $R^2 = 0.962$). Bulked samples had average concentrations within ±6%, average error of –1.5% and average absolute error of 12%.

## Advantages and Limitations

Rainfall simulators allow controlled experiments, with thorough, timely collection and storage of high quality samples. This is generally difficult and expensive during natural rainfall events. For studies of pesticides, where preservation of samples is important, samples can be collected by hand and refrigerated, filtered, extracted etc. immediately. Detailed time series of runoff concentrations, or flow-weighted average samples, can be obtained. In contrast, measurement of pesticides in runoff from natural rainfall plots and catchments is more problematic and generally use either flow-splitters to obtain event flow-weighted averages, hand sampling during events, limited sampling and some form of extrapolation/estimation, or automatic pumping samplers generally with refrigeration, an expensive option but still with a mixed record of success. Even where a series of samples are collected using pumping samplers, flow-weighted composite samples are often prepared to reduce the cost of analysis.

Another advantage of rainfall simulators is that a reasonably large number of treatments can be compared under similar conditions, which is difficult and expensive using catchments. Factors such as time between pesticide application and rainfall occurrence can be controlled and studied as an experimental variable. Rainfall simulators are also useful for demonstration and extension, in part because they are “visual”. Several treatments can be run side-by-side under one simulator, allowing a direct comparison between, say, stubble retention treatments. Getting immediate pesticide data is more difficult but is possible using immunoassay methods (*26*).

Disadvantages of rainfall simulators include the logistics involved (number of people needed, water supply) and issues of scale/plot size. However, the most important issue is that rainfall simulators do not define the long-term average and distribution of runoff, erosion and pesticide losses. They apply rainfall

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events and thus define the responses to similar events. Rainfall simulator results have been successfully translated to long-term times series, probability distributions and averages, using simulation models, as discussed below.

## 'Validation' of Rainfall Simulators

Simplistic approaches to comparing and using data from rainfall simulators to represent hydrology, erosion and water quality under natural rainfall and for larger scales are problematic. However, there has been considerable success using models that represent scaling-up of physical processes. Firstly, it is important to be able to transpose hydrology and erosion from simulators to catchments. Silburn and Connolly (*27*) determined parameters for a layered infiltration model from rainfall simulator plots, with treatments designed to reveal the hydraulic properties of the various soil layers. These infiltration parameters, when used in a spatially distributed hydrologic model, gave good estimates of runoff hydrographs for large simulator plots and under natural rain for spatially uniform (*28*) and complex (*29*) catchments.

Similarly, for soil erosion, parameters derived for a physically based erosion model from small (interrill) and large (rill) rainfall simulator plots, provided good predictions of event soil losses measured on hillslope plots and catchments (*23,30,31*), so long as the same process dominated. Simpler models did not adequately predict these field soil losses using the same simulator data (*30*).

Wauchope (*2*) defined pesticide runoff for 'critical events' (within 2 weeks of pesticide application, >10mm rainfall, 50 % of which becomes runoff) and 'catastrophic' events (first events soon after application), and considered rainfall simulator events where severe rainfall is applied soon after application of a pesticide as 'catastrophic'. He also considered that small plots gave reasonable agreement with large fields as far as concentrations are concerned, but can overestimate long-term losses from larger fields by a factor of two. Rainfall simulators have often been used to apply storms that exceed the 'critical' event, which leads to large losses. This choice made by the rainfall simulator scientist is probably related to the history of use of simulators in erosion research, where large infrequent storms cause the majority (>80%) of soil loss.

Rainfall simulator plots have often been run soon after pesticide application, resulting in large losses in runoff (i.e. as % of applied) and leading to events classed as 'catastrophic'. For example, White *et al.* (*18*) found losses of 17% and 7.3% of applied atrazine at 1 and 96 hr after application, respectively. Losses of this magnitude are not unheard of in catchment studies (*2,32*) and probably represent well what would happen on a catchment if a large runoff event occurring very soon after spraying. Time of the applied storm after spraying is a matter of choice by the rainfall simulator scientist. The main reasons studies are often run soon after spraying are probably logistical

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(finishing the study before natural rain or farm operations disturb the site) and, given the effort involved and uncertainty in dissipation rates, concerns that concentrations will fall below detection limits. This tendency to overestimate pesticide runoff loads can be discounted using the pesticide runoff framework.

Rainfall simulators can also help inform more complex models. For example, Connolly *et al.* (*33*) used GLEAMS to model runoff, sediment and pesticide transport from irrigated cotton fields. Important parameters relating to effects of cover, sediment size and pesticide partitioning (not studied at the field scale) were derived from the simulator studies of (*24,25*). The model predicted field behavior well, although some parameters had to be altered at larger scales.

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## Pesticide Runoff from Rainfall Simulators and Catchments

Data from rainfall simulators and catchments that are directly comparable are uncommon. However, White *et al.* (*34*) published such a study, though they did not interpret it as we have here. They measured runoff of 2,4-D from a small, cultivated catchment in Georgia, and measured concentrations in soil (0-5mm) after 2,4-D application for one year. Simulated rainfall was applied to three 30.2 $m^2$ sub-plots at 1, 8 and 35 days after 2,4-D application, at a high intensity (165mm/h for 30 min). We determined soil concentrations for storms and for simulator plots from days after application by interpolating between the measured soil concentration data, similar to (*7*). Two aspects of the data are of interest here, namely, comparison of rainfall simulator data with catchment data, and comparison of soil and runoff concentrations.

Mean concentrations of 2,4-D in runoff from simulator plots and the catchment fit the same first-order relationship with days after spraying (Figure 5), indicating that the rainfall simulator provided a reasonable estimate of pesticide runoff from the catchment. The concentration from the simulator are between the concentrations from the catchment for two storms at day 34. The maximum concentrations in runoff from the simulator plots also agree with maximum concentrations measured from the catchments when considered as functions of days after application (Figure 5).

Mean runoff concentrations for the rainfall simulator plots and the catchment storms relate well to soil concentrations (Figure 6) and cluster around the equations of Silburn (*6*) and Leonard *et al.* (*7*). The lower runoff extraction at one day after spraying may be related to greater leaching out of the runoff mixing layer and little contribution from sediment phase transport, as the effective partitioning coefficient (calculated from the concentrations in water and sediment) was much lower than at later times.

Data from rainfall simulator plots of Trichell *et al.* (*17*), for 2,4,5-T, picloram and dicamba (acidic herbicides like 2,4-D) on fallow plots, also have a reasonably close relationship with the other data and equations in Figure 6.

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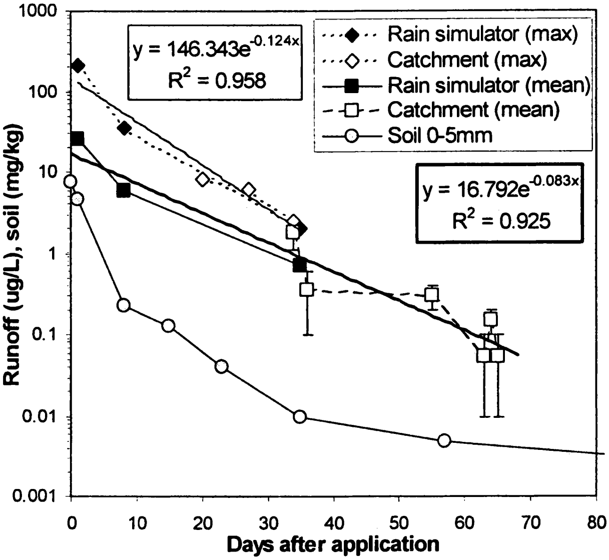

*Figure 5. 2,4-D in runoff from rainfall simulator plots and a catchment, through time after application (data from 34). Concentrations are event means (symbol), maximum and minimum (error bars), and maximum annual ('max').*

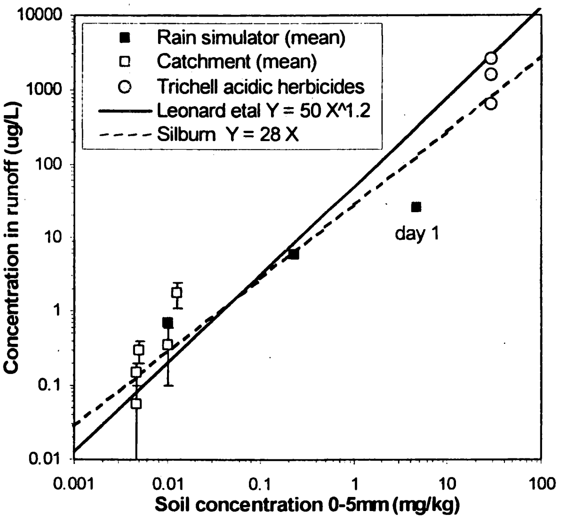

*Figure 6. Mean concentrations of 2,4-D in soil (0-5mm) and in runoff from simulator plots and a catchment (34), and for 2,4,5-T, picloram and dicamba from (17), compared with (7) Y=50 X^1.2 and Silburn (6) (Y = 28 X).*

These data illustrate that the runoff concentrations were very high because the soil concentrations were very high, not because these herbicides have an intrinsically high runoff potential. Taking the concentrations in runoff on face value gives a false picture of the runoff potential of these pesticides.

Silburn (*6*) found that the herbicide data of Leonard *et al.* (*7*) (for atrazine, cyanazine, diphenamid and propazine) from catchment also coincided with the data of Baker and Laflen (*35*) from 1.5 by 9.1m rainfall simulator plots (atrazine, propachlor and alachlor) when plotted in the framework. The rainfall simulator plots were run 7-11 h after spraying whereas runoff events on the catchments occurred at various longer times after spraying. This was largely accounted for by the soil concentrations. However, in catchments where erosion rates were considerably greater (e.g. *32*, slopes of 10-15%) runoff concentrations of atrazine and propachlor were three to four times higher (per unit of soil concentration) than in the above studies. Pesticide runoff data from three rainfall simulator studies by Silburn (*6*) (Y = 28 X) also coincided with data from catchments (*7,34*) and plots (*34,35*) when plotted in the framework.

## Pesticide Partitioning in Runoff

Rainfall simulators can also be used to study partitioning of pesticides between water and sediment under field conditions. Partitioning is important in determining mobility and environmental fate. The data most commonly available describing partitioning is the soil sorption coefficient ($K_D$ as define in (*1*)) measured in laboratory studies. Partitioning in runoff ($K_P$) may differ from that in soil, as runoff is associated with desorption (whereas $K_D$ is often measured for adsorption), and greater water-solid ratios and longer times of soil-pesticide contact. $K_P$ values measured in runoff are generally greater than soil-water $K_D$ values (*6,8,35*) and increase with time of contact with soil (*6,36*). In a rainfall simulator study on a black clay soil, with pesticides with a range of properties, $K_P$ increased with time after application for all pesticides (Figure 7) and was often greater than suggested in the literature (*6*). Thus supposedly weakly sorbed pesticides may be more manageable by sediment control than expected from their $K_D$ values.

## Pesticides Persistence and Washoff from Plant Canopies

Rainfall simulators are useful for studying washoff of pesticides by rainfall from plant canopies (37,38) and crop residues (39). This may relate to the water and pesticide contribution to runoff under the canopy/residues, or to the rain-fastness of the pesticide. Concentration of pesticides in washoff water from both plants and crop residues typically decline rapidly with amount of applied rain.

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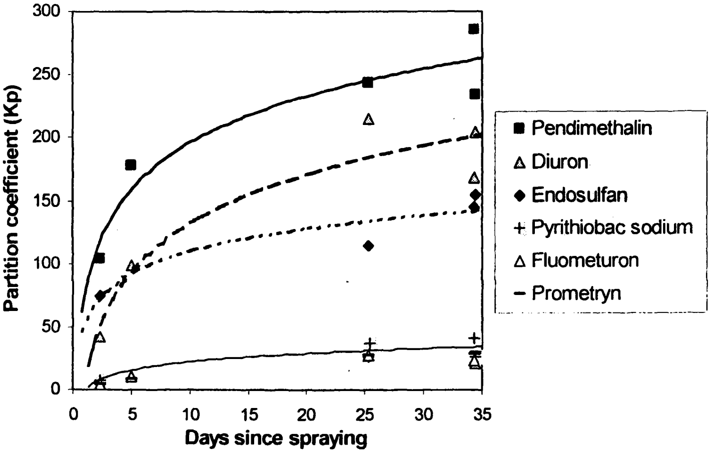

*Figure 7. Partitioning of pesticides between water and sediment in runoff from rainfall simulator plots, at various times after application (6).*

The first few mm of rainfall will wash off a large proportion (e.g. 50%) of the total pesticide washed off (*37-38*). There are no intrinsic problems with the small scale of rainfall simulators for these studies. Washoff is related to the amount of rain, not the intensity (*38*), so choice of rainfall intensity does not seem to be problematic. As with studies on soils, dissipation should be studied simultaneously, to enhance the knowledge gained and interpret the results.

Silburn *et al.* (*40*) studied washoff of endosulfan from cotton plants using a rainfall simulator at four times after application. Wash-off water was collected from three gutters under the crop, with the aim of collecting a large volume and a good spatial average of washoff water. Endosulfan in plants and in washoff declined rapidly after application (Figure 8). Concentrations in washoff declined rapidly during rainfall, with a similar pattern through time on the four days after application. Endosulfan washoff was similar to EPN, less rapid than fenvalerate and permethrin, and more rapid than toxaphene, compared with (*38*).

## Conclusions

A pesticide runoff framework, which accounts for pesticide application rate and dissipation prior to runoff by using pesticide concentrations in surface soil, was useful in comparing different pesticides and studies. It indicates that rainfall simulators generally produce runoff concentrations similar to those from small catchments and plots under natural rainfall, once the soil concentrations are considered, so long as the soil erosion rates are not drastically different. Rainfall simulator studies also provide useful insights into washoff from plants and

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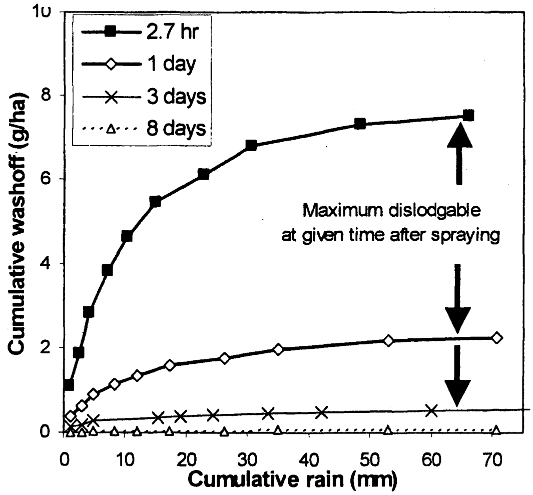

*Figure 8. Washoff of endosulfan (alpha+beta+sulfate) from cotton plants by rain, at four times after spraying. (40)*

partitioning between sediment and water phases in runoff under field rainfall conditions. Such partition coefficients increases with time after spraying and are often greater than laboratory soil sorption values.

## Acknowledgement

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## Chapter 9

# Progress in the Development of Biosensors for Environmental and Human Monitoring of Agrochemicals

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Monitoring studies for toxic substances in environmental and biological samples are essential for evaluating risk to human and environmental health. Immunoassays are one method that can satisfy analytical demands for high sample loads requiring high sensitivity because they are rapid, can be adapted to high throughput or biosensor formats and can be adapted for small sample sizes. A description of progress in the field of immunoassays and the application to biosensors in general is presented with a focus on research from this laboratory.

In order to make rational decisions on the selection and use of agrochemicals, data from environmental fate and target and non-target organism exposure as well as human exposure are needed, in addition to continuing monitoring studies to validate these decisions. Environmental monitoring studies of the residues of toxic chemical contaminants can show how residues influx to humans from environmental media such as air, water, food, consumer products, soil and settled dust. These studies answer questions about source for exposure (anthropogenic/non-anthropogenic, area/point, and indoor/outdoor); the transport/carrier medium; the exposure pathway (eating, breathing, touching); and how long the residues exist *(1)*. In evaluating human exposure,

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

biomonitoring studies assess the nature of the health hazard; how many people are exposed; to how much; identify the exposure pathway (inhalation, ingestion, or dermal contact); determine the residual concentrations in people; define the relationship to human health effects; and identify special populations or individuals that may be exposed.

These two types of studies help provide the most complete exposure information for toxicologists and epidemiologists to evaluate the potential for adverse health effects on humans or other non-target organisms and the environment. The data can be used to select strategies to maximize crop production with proper planning and implementation of pesticide/pest management practices while minimizing adverse exposures to humans and the environment. Such complex monitoring studies require hundreds or thousands of samples to obtain sufficient data. Thus, there is a need for rapid, quantitative, high throughput analysis methods.

Gas chromatography (GC) or high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) are the first choice of methods for large scale screening studies for multiple analytes for both biomonitoring *(2, 3)* and environmental monitoring *(4)*. These techniques are well documented and give accurate results. Along with instrumental methods, affinity-based analyses can play an important role in high-throughput applications. Such affinity-based assays rely on biological reagents such as enzymes, DNA/RNA aptamers and antibodies or chemical reagents such as molecularly imprinted polymers.

Antibody-based immunoassays have been proven to be a rapid, sensitive and cost effective analytical tool for routine monitoring *(5)*. They are particularly well-suited to analysis of certain substances that are more difficult to analyze with GC or HPLC because of large molecular mass, thermal lability, low volatility or lack of a distinct chromophore. Antibodies in immunoassays act as a receptor (detector) for the analyte of interest. Binding occurs through hydrogen bonding, hydrophobic bonding, electrostatic, van der Waals forces and by the degree of complementarity between the antibody and the antigen, making it an ideal method for compounds with physical and chemical properties that are difficult to analyze by other instruments *(6, 7)*.

Moreover, immunoassays can be formatted to use only a small volume of sample, are adaptable to high throughput methods using the 96- or 384-microwell plate or current autoanalyzer systems and are particularly useful as the primary screening method to reduce a large sample set by avoiding the need to completely work-up negative samples. In this scheme, conventional instrumental analyses with GC and/or HPLC that can then focus on the more interesting and positive samples *(8)*. But immunoassays can also be used as the primary means of analysis as exemplified by a study carried out in our laboratory in which paraquat was measured in urine collected from farm workers exposed to the herbicide in order to study the relationship of exposure to agricultural lung disease *(9, 10)*. The paraquat immunoassay combined with a cation exchange solid phase extraction (SPE) method for urine extraction was demonstrated as an

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excellent tool with the advantages described above *(11)*. Immunoassay methods are accepted by regulatory agencies as demonstrated by the validated method for the natural insecticide spinosad in ruminant commodities that is set for inclusion in the US Food and Drug Administration Pesticide Analytical Manual Volume II *(12)* as well as methods described in the Environmental Protection Agency Residue Analytical Methods *(13)* and those submitted to the EPA Office of Pesticide Programs as part of registration packages.

As an improved tool, biosensors result from the association of a sensitive biological element with a transducer, which converts the biological signal into a measurable physical signal (Figure 1). The biological element may be an enzyme, antibody, cell, DNA, DNA/RNA aptamer or receptor.

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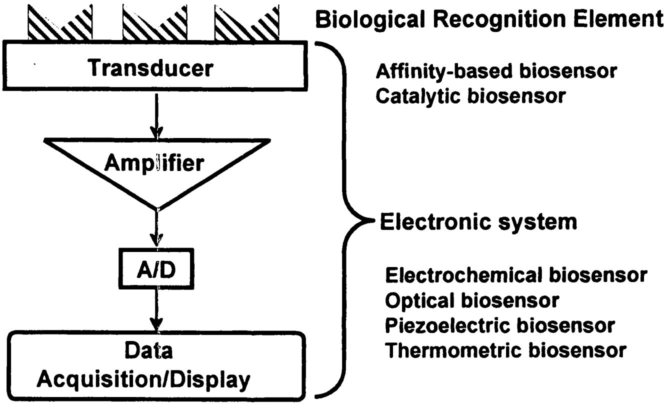
Biological Recognition Element
Transducer
Affinity-based biosensor
Catalytic biosensor
Amplifier
Electronic system
A/D
Electrochemical biosensor
Optical biosensor
Piezoelectric biosensor
Thermometric biosensor
Data
Acquisition/Display

*Figure 1. Schematic of a biosensor*

A recent review describes the applications of these various types of elements in biosensors for environmental monitoring *(14)*. The transducer is in close proximity or is integrated with an analyte-selective interface *(15)*. The transducer signal may be mass (microbalance), electrochemical or optical (surface plasmon resonance, fluorescence, chemiluminescence). Descriptions of types of signal transduction for biosensors is given in Suri *et al.* *(16)*. Immunosensors are a specialized type of biosensor, which utilize antibodies for detection. Antibody-based sensors can provide continuous, *in situ*, rapid and sensitive measurement based on the conventional immunoassay.

To develop an integrated affinity-based biosensor requires research into the components of the immunoassay (haptens, binding molecules, formats, labels) as well as the the detection system, the reagent delivery system, etc. Each of these alone is a large area of research. We review briefly here advances in selected areas of biosensor research for agrochemicals.

## Assay Improvements

Immunoassays for small molecule environmental toxicants, such as pesticides, are developed with the processes of synthesis of haptens as immunogens and coating antigens. Using the immunogens, antibodies are produced. The antibodies are screened against a library of competitor haptens (coating antigens). Assays with high sensitivity and selectivity are optimized to avoid interferences and perform in the matrix of interest and finally the assay is validated with real analytical samples. An overview of this procedure is described in Suri *et al.* *(16)*.

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### Hapten design approaches

Since small molecules, like environmental toxicants, are not immunogenic, mimics of the toxicant that contain functional groups for use in coupling to carrier molecules (e.g. peptides, protein, polymers) are synthesized, coupled and used to immunize animals to generate antibodies. For immunizing a close structural mimic of the toxicant of interest is required (immunizing hapten). In analyzing the toxicant, an immunoassay is usually competitive. That is, the toxicant (analyte) competes with an analyte mimic coupled to a carrier molecule that is used in the assay (competitive hapten). The more effectively the analyte can compete, the lower the detection limit of the assay. Many assays use the same hapten for immunizing as for competition in the assay. The drawback is that the antibody may have a higher affinity for the analyte mimic, than for the free analyte. One strategy to overcome this drawback, is to synthesize other mimics of the analyte (heterologous haptens) that will bind with less affinity to the antibody. One approach is to change the bridge chemistry (e.g. handle length or coupling chemistry). For example with the herbicide atrazine, changing the bridge length from two carbons to six carbons resulted in a more sensitive assay *(17, 18)*. One can also replace part of the structure (e.g. with an isoster, S/Cl or to substitute another atom with less hydrogen binding capacity such as a carbon for a nitrogen) or to use a partial structure *(17)*. This strategy was used successfully for the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Substituting phage-displayed peptides as mimics of the hapten is a recent approach. The phage display system produces randomly generated amino acid sequences fused to coat proteins of the filamentous phage *(19, 20)*. By utilizing the phage display method, a vast repertoire of peptides can be expressed as libraries. The phage libraries can be rapidly screened by enriching for specific clones by repetitive rounds of affinity selection. The peptides are typically 12-16 amino acids and can be selected with a high affinity *(21)*. Use of peptide haptens in assays for molinate and atrazine resulted in detection limits that were similar (for molinate) or lower (atrazine) than the assays with the corresponding chemical hapten *(22)*.

## Improved binding molecules

Improvements to the sensitivity, selectivity and/or ruggedness of the assay have also been approached by using modified antibodies (antibody fragments, recombinant antibodies, sol gel-encapsulated antibodies), biological antibody replacements (peptides, aptamers) or chemical replacements (molecularly imprinted polymers). The most desirable antibodies for small molecule analysis have a high affinity for the analyte of interest, are stable under the conditions of analysis, stable to potential matrix interferences, exhibit low background in the assay and have limited cross reactivity to non-analyte molecules. Antibodies are immunoglobulins that consist of four fragments (two identical light chains and two identical heavy chains). The fragment of antibodies that bind antigens ($F_{ab}$) consists of variable regions of the light and heavy chains linked by a disulfide bridge through a constant region associated with each chain. $F_{ab}$ fragments are produced by enzymatic cleavage of the antibody molecule. $F_{ab}$ fragments are sometimes desirable as their use produces lower background in some formats, but the fragments often lack the avidity of the whole molecule *(23)*.

Using recombinant techniques, the variable regions of the light and heavy chains can be synthesized (without the associated constant region) and their respective N- and C-termini linked by a peptide bridge. These antibodies are termed single chain fragments of the variable region (scFv). Advantages to recombinant technology are the ability to systematically improve the primary binding site and to create fusion proteins. Fusion proteins contain the primary antibody protein fused to a reporter protein. Such a fusion protein has been made for the detection of atrazine using the enzyme alkaline phosphatase *(24)* and for picloram using a fluorescent protein *(25)*. Fusion proteins that facilitate separation in a homogeneous assay for atrazine have also been reported *(26)*. Reviews of the development of the field of recombinant antibodies for environmental analysis is presented by Kramer and Hock *(7)* and Yau *et al. (27)*. Application of assays using recombinant reagents for pesticide environmental analysis have been reported for atrazine *(7)* and simetryn *(28)*.

Similar to hapten strategies described above, phage displayed peptides can also mimic the antibody. To our knowledge there are no reports utilizing phage display peptides as binding molecules for small molecules for environmental analysis. However, there are a large number of reports for phage display binding to peptides and larger antigens such as cell surface antigens or receptors. Further details of this technology and uses for peptide binding molecules can be found in a review by Kehoe and Kay *(29)*. For most environmental immunoassays, the analyses are in a competitive format, rather than a sandwich format. One novel use for peptides would be to detect a small molecule bound to it's capture antibody. In this way the assay would more closely resemble a sandwich assay and would be a way to more easily utilize array technologies *(30)*.

Nucleic acids are typically used as probes to hybridize with and detect RNA and DNA target sequences that are complementary. Recently, aptamers, short

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synthetic DNA and RNA sequences, are being used as ligands to bind to targets other than nucleic acids with high affinity and specificity. Although typically used to detect peptides and proteins, aptamers have been developed to detect compounds as small as ethanolamine *(31)*. They are especially attractive as analytical reagents because they are more temperature stable and more easily produced than antibodies. On the other hand, their binding ability depends on their folding and three dimensional structure which is susceptible to incubation and buffer compositions *(32)*. Aptamers are commonly 'synthesized' via a combinatorial chemistry technique known as systematic evolution of ligands by exponential enrichment *(33)*. Resulting aptamers have application in affinity separations, as the binding element in biosensors or as signaling molecules (i.e. molecular beacons) *(34)*. A biotinylated RNA aptamer made against L-2-phenoxypropionic acid was bound to streptavidin-coated silica to make a solid support for capillary electrophoresis. The capillary column proved robust in that more than 700 injections at concentrations of acetonitrile of 5-30% and temperatures of 5-40 °C showed no differences in retention time and peak shape for several herbicides in the aryloxyphenoxypropionic family *(35)*. In addition, the column was capable of separating the enantiomers of the herbicides. Recently, aptamers for cocaine have been applied to a label-free biosensor. One end of the aptamer is immobilized on a gold electrode surface. The other end is tagged with methylene blue. When a cocaine molecule is bound by the aptamer, the methylene blue-tagged end comes in proximity to the electrode surface generating an electric signal. With the ease of making aptamers, this system should be generally applicable to detection of pesticides in environmental or biological samples *(36)*.

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## Improved labels

Classically, labels to detect antibody binding have been radiometric, colorimetric, or fluorescent/chemiluminescent. Labels that can be detected sensitively, with high signal:noise ratio, that are stable and robust are ideal. Fluorescent labels are generally thought to be detected with greater sensitivity than colorimetric labels, but they can subject to interference from background fluorescing materials, photobleaching, short half lives and narrow Stoke's shifts. An improvement would be to use labels that fluoresce at longer emission wavelengths and/or chemiluminescent materials. Detection of such labels would be away from wavelengths where naturally fluorescing materials occur, thus reducing background interference. We have focused on the lanthanide europium oxide ($Eu_2O_3$) nanoparticles as fluorescent labels. There is a large body of literature using the europium ion as a reporter for immunoassay when complexed in a chelate, but few reports using the lanthanide alone. The lanthanides have large Stoke's shifts, sharp emission peaks, emission at a wavelength generally free of interference from natural biological fluorescence and a long half-life to facilitate the use of time resolved mode, further increasing the signal to noise ratio.

In one example of our work, inorganic $Eu_2O_3$ nanoparticles were used as a novel fluorescent reporter in an immunoassay for 3-phenoxybenzoic acid (3-PBA) a breakdown product of a number of pyrethroid insecticides *(Ahn et al., unpublished data)*. The surface of the particles was functionalized with either an aminopropyltrimethylsilane using a microwave coating method or with a simple aminopropylsilatrane (APS) coating method under aqueous conditions. As shown in Figure 2, $NH_2$-functionalized particles were coupled to 3-PBA for a competitive immunoassay.

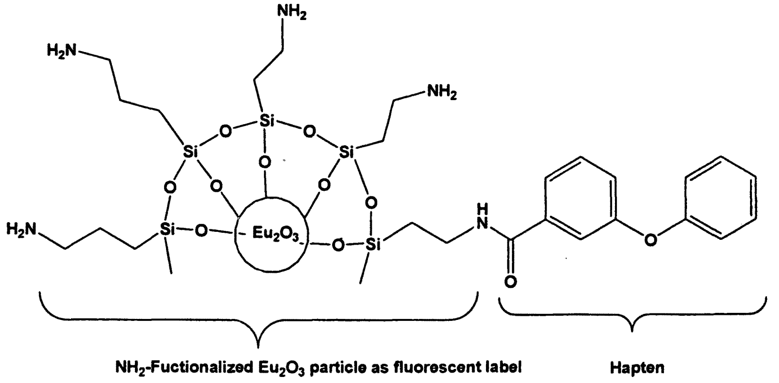

*Figure 2. Putative structure of europium particle-labeled hapten*

The $Eu_2O_3$-fluorescent immunoassay using a magnetic separation technique and paramagnetic secondary antibody in the assay procedure remarkably improved the sensitivity, compared to the conventional microplate ELISA (*37*). However, an assay for 3-PBA-glycine using particles coated by the microwave method was not as sensitive as the conventional microplate ELISA (*38*). This suggests that the coupling technique is important to the performance of the assay, but further research is necessary. The fluorescence immunoassay is simple and rapid, required no washing steps and no enzyme conjugates, making it of significant utility for a high-throughput assay.

## Improved Throughput Techniques

### Immunoaffinity Chromatography

One approach to improving throughput for agrochemical analysis is to adapt immunoassays to combine cleanup and detection into one device. An immunoaffinity chromatography (IAC) is based on a solid-phase extraction principle, but utilizes the selectivity of antibody to isolate and concentrate a

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specific antigenic analyte from a sample matrix. Immunoaffinity matrices are developed by chemically coupling the ligand (a pure antigen, an antibody or an anti-antibody) to a suitable matrix such as sepharose or agarose. An IAC generally enhances the detection sensitivity, recovery rate and analytical time. The resulting extracts for the subsequent detection is generally cleaner than those of the conventional solid-phase extraction methods. They consist of less interfering substances leading to less interfering peaks in the chromatographic detection, hence improving the detection sensitivity.

Coupling immunoaffinity chromatography to an instrumental system allows a fully automated system. This design combines the advantages of high selectivity and throughput capacity of immunoaffinity chromatography with that of high accuracy/precision of the instruments. Some successful examples include analysis for β-agonists *(39)*, benzene/toluene/ethyl benzene/xylene *(40)*, isoproturon and fluoroquinolones *(41)*.

A disadvantage is that the commercial immunoaffinity columns only warrant a single use, although re-generation for repeated usage may be possible *(42)*. Reusability is dependent upon various factors including the type of antibody, specific antibody-antigen interaction involved and elution conditions being used. Immunoaffinity chromatography has been re-used as many as ten times without significant loss of performance.

One way to stabilize antibodies to make them more reusable in IAC applications is to encapsulate them in a matrix such as a sol-gel. Sol-gel encapsulated antibodies retain their binding capacity and are being widely investigated for application in biosensor development *(43)*. For pesticides, sol-gel-based immunoabsorbents have been used for the detection of the herbicide isoproturon *(44)*, triazine herbicides and the insecticide malathion *(45)*, and sulfonylurea herbicides *(46)*.

Unlike immunoassay, an IAC requires a fairly large amount of antibody to be effective - at least 0.1-1 mg per column. Thus monoclonal antibody is the current general source of antibody if a bulk production is intended. However, as mentioned above, other binding reagents such as recombinant antibodies, phage displayed peptides or aptamers could be used. Like immunoassays, immunoaffinity columns perform best within their expiration dates and their shelf life is relatively short (up to one year in most cases). Because they are still produced in batches, there may potentially be some batch-to-batch variation, which may require an additional validation of every new batch being produced.

IAC application in the area of food contaminants other than pesticide residues has been active and a number of IAC are now commercially available for this purpose. Environmental application of IAC has been slow possibly due to skepticism within the general analytical community. Also the availability of effective sample preparation methods for environmental residues could have impeded the development of IAC for environmental application. However, advances in antibody stabilization and miniaturization, make the IAC approach attractive for biosensor development.

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## Immunochromatography

Immunochromatography combines the power of immunochemistry and chromatography on a membrane. Membrane or test strip based assays can exist in various forms such as dipstick, lateral flow device and vertical flow device. These devices are designed to augment the performance of immunoassays in a more user-friendly fashion, delivering results in minutes with either no or very simple extraction. The greatly enhanced portability in ready to use test kits has made this technology very attractive as a decision support tool to industries and regulators in recent years. The advantages of such rapid systems are ease of handling, no washing procedures, no substrate required, results can be obtained within minutes and results are permanent (when visualization system other than enzyme-substrate system is used). However, the main drawback of the current rapid systems is low sensitivity compared to their alternative formats.

A dipstick assay with an immobilized antibody works on the same principle as in a competitive immunoassay of a microwell plate, a membrane strip is first spotted with a hapten-specific antibody and a label-specific antibody such as an anti-enzyme antibody. The hapten-specific antibody must be able to interact with the sample analyte and the competitor hapten within a short contact time. The tracer-specific antibody, on the other hand, must be capable of binding with the tracer to serve as a control or a reference *(47-50)*. When performing the assay, the strip is immersed into a sample solution containing a known quantity of labelled competitor/tracer to allow competition between the analyte and competitor to occur. When no analyte is present, maximum color would develop. When the analyte is in excess of the labelled competitors, the test dot will remain invisible when the substrate is added. A dipstick assay with immobilised antigen can be performed with a similar approach except that the membrane will be immobilized with a hapten competitor (hapten-protein conjugate) and an anti-species antibody (as a control). The system would be visualized by a labelled hapten-specific antibody coupled to an enzyme. The dipstick assays have been developed for carbaryl *(51)*, atrazine *(52)*, terbuthylazine *(53)*, ametryn *(54)*, fenthion *(50)* and 2,4-dichlorophenoxyacetic acid *(55)*.

Unlike a dipstick, a lateral flow device involves the transport of sample analyte and labelled-antibody through a test strip to the testing zones by capillary action. The immobilization is generally achieved by striping to obtain fine lines (generally 1mm thick). During the transport through the membrane, the sample analyte is given sufficient time to compete with the labelled protein, while larger unwanted molecules are hindered by the small membrane pores. Such a separation mechanism helps to reduce interference to the required immunoreactions at the upper part of the test strip, thereby improving the performance compared to a dipstick *(56, 57)*. The adsorbent pad on the lateral

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flow device, where the sample is applied, also acts like a filter and further helps to reduce interference moving with the antibody. The lateral flow or flow through devices have been developed for 2,4-dichlorophenoxyacetic acid *(58)*, alachlor *(56)*, carbaryl *(57)* and endosulfan *(57)*.

A typical vertical flow device consists of a plexiglass base, an absorbent layer, membrane coated with antiserum and a plexiglass lid with holes for introducing samples *(59-63)*. Membrane pieces (typically of 3 x 3 cm$^2$) are firstly striped with hapten-specific antibody and tracer-specific antibody. Upon blocking, the membranes are fixed into the test device. Standard or sample solution, wash solution, labelled competitor hapten solution and wash solution are then dropped sequentially onto the membrane with a 1-1.5 min interval between each solution. This permits the liquid to be adequately absorbed by the membrane and interact with the immobilized components. Any excess or unwanted components will be absorbed by the absorbent layer underneath.

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When an enzyme is used as a tracer, substrate solution will then be added for color development. The incubation time for this process is approximately one minute *(60)*. The reaction is subsequently stopped by washing with a surfactant such as Tween 20. The total test time for this assay is typically around 10 min *(60, 62)*. The absorbance of colour *(63)* or fluorescent complex *(61)*an be directly measured by a reflectometer. If colloidal dye, colloidal gold or luminal particles are used in place of an enzyme-substrate system, imaging system such as FluoroImager can be used for visualization and subsequent quantification. Assay time with such visualisation systems can be as short as 2 min. The immobilization of antibody, flow techniques and rapid visualization lend itself the to development to the design of lab-on-a-chip type biosensors.

## High throughput platforms

Another approach to improving throughput is to adapt existing environmental immunoassays to current clinical automated, high throughput platforms. We have adapted a competitive chemiluminescent assay for the detection of 3-PBA based on polyclonal antibodies with an automatic Bayer ACS:180 immunoassay analyzer system. The chemiluminescent acridinium ester label was linked to the 3-PBA-BSA conjugate. The optimized competitive immunoassay format showed high sensitivity (IC$_{50}$ values of 0.3 µg/L) for 3-PBA, compared to that (IC$_{50}$=2 µg/L) of the conventional microplate ELISA. In this method, the competition step is performed offline, then the immunoassay was performed by the fully automatic analyzer using paramagnetic secondary antibody for the separation of immunocomplex and non-immunocomplex. A mixed-mode solid phase extraction (C8 and anion exchange) used to prepare samples prior to analysis to reduce matrix interferences increased the assay

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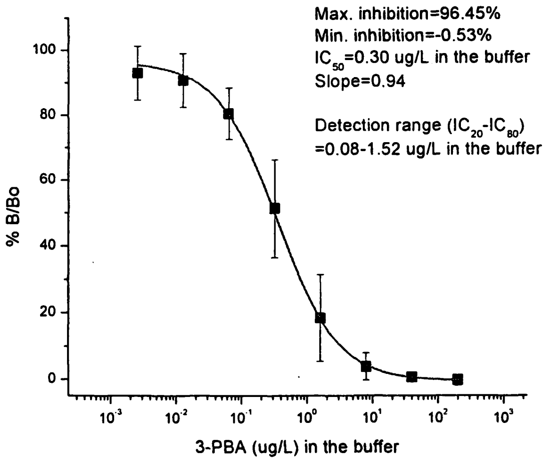

*Figure 3. Calibration curve to 3-PBA in the ACS:180 chemiluminescent assay format.*

sensitivity, resulting in measurement as low as 1 µg/L urine of 3-PBA with a limit of detection (LOD) of 0.08 µg/L in buffer (Figure 3).

The assay on the ACS:180 provided the first results within 15 min, and yielded around 130 results per hour. The analyzer combined with the highly sensitive chemiluminescent acridinium label, magnetic particle separation, and the automatic processes will support high throughput assay for monitoring human exposure to pyrethroids by measuring 3-PBA *(Ahn et al., unpublished data)* and can easily be applied to analysis of other pesticides using reagents already developed for the classical enzyme linked immunosorbent assay. The automated chemiluminescent immunoassay has excellent advantages in terms of sensitivity, rapidity, and simplicity for application to monitoring studies.

Multiplex fluorescence bead-based immunoassay technology has recently been introduced as a high throughput method for multianalyte screening. When particles are used, the speed of an assay compared to a microtiter-well plate assay can be accelerated because of the mobile property and the larger surface area *(64)*. The small size of polymeric and inorganic particles emitting light or a sensing element homogenously distributed throughout the particle can be used as label markers, and satisfy criteria for high brightness, a unique signal, and relatively low cost *(64)*. The microparticles encoded with different amounts of a dye can be distinguished with the flow cytometric assay *(65)*. The particle assay can be convenient to separate the free and bound complex. It can efficiently reduce interference caused from sample matrices by separation with filtration or magnetism when the antibodies are attached to paramagnetic particles. Unlike the ACS platform that is one sample, one analyte, the mutiplex bead assay is one sample, multiple analyte format. The fluorescent flow cytometric assays

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combined with the use of different sized polystyrene microbeads allows one to perform specific and quantitative immunoassays. This technique was demonstrated for the detection of target analytes, glyphosate, atrazine and the mercapturate of metolachlor *(66)* in urine of exposed farmworkers. The least detectable dose of glyphosate, atrazine and metolachlor mercapturate in urine were 0.9, 0.7 and 0.3 ng/mL respectively similar to or lower than determined by HPLC. It is possible to expand this technique up to 100 analytes according to the manufacturer, but one must keep in mind that cross-reacting analytes can complicate the interpretation of results. One advantage of this method is that it is currently used to detect biomarkers of effect such as cytokines as indicators of inflammation. It is a simple step to combine reagents for the detection of pesticide metabolites as biomarkers of exposure and from one sample have data to help in the assessment of the continuum from exposure to effect in humans.

## Microarrays

Protein microarrays have the potential to play a fundamental role in the miniaturization of biosensors, high throughput drug screening, clinical immunological assays, and protein-protein interaction studies *(30, 67)*. A quantititative microarray for atrazine, and the metabolite of dichlobenil, 2,6-dichlorobenzamide has been reported. The microarray was constructed using a spotter to place hapten conjugates in discrete spots on a glass slide. The slide was then subjected to UV light to covalently immobilize the conjugate. A fixed abount of Cy5-labeled antibody for each analyte was added along with the sample or standard. The lowest detection limits for 2,5-dichlorobenzamide and atrazine were 1 and 3 ng/L, respectively *(68)*. As in a typical ELISA, the free analyte competed with the immobilized conjugate for a fixed number of antibody binding sites. After a wash step, bound antibodies are visualized by confocal microscopy. Like many microarrays, the one described here was visualized and analyzed using organic fluorescent dyes *(69)*.

To overcome photobleaching and spectral overlaps we applied a new type of fluorophore, crystalline europium-doped gadolinium oxide (Eu:$Gd_2O_3$) nanoparticles, as labels in protein and antibody microarrays *(70, 71)*. The Eu:$Gd_2O_3$ nanoparticles were synthesized by spray pyrolysis and they offer narrow red emission, large Stokes shift, photostable laser-induced fluorescence with a long lifetime (1 msec) *(72)*. Recently, the nanoparticles were successfully applied as reporters in a competitive fluorescence microimmunoassay for 3-PBA *(73)*. The nanoparticles were functionalized with reactive amino groups by poly-L-lysine encapsulation The PL-encapsulated particles were covalently conjugated to the specific anti-PBA antibody. The microarrays were fabricated by direct microcontact printing *(69)* of the coating antigen BSA-PBA in line patterns (10 x 10 μm) on glass substrates. The nonprinted surface was blocked with BSA-fluorescein.

The competitive immunoassay for PBA was carried out with a preincubation of the labeled antibody (anti-PBA IgG-PL-Eu:$Gd_2O_3$) with the analyte. Then the glass substrates (printed BSA-PBA/blocked BSA-fluorescein) were incubated with these solutions for 1h where the non-saturated binding sites of the labeled antibody bind to the printed PBA. Confocal fluorescence microscopy combined with internal standard (fluorescein) calibration was used for quantitative measurements. Representative fluorescence images of the patterns obtained for the PBA concentrations tested are shown in Figure 4a. The yellow strips correspond to the internal standard (fluorescein) fluorescence which has similar intensity for the four substrates. Increasing PBA concentration leads to smaller amounts of nanoparticles bound on the printed BSA-PBA strips and therefore to a decrease in the red Eu:$Gd_2O_3$ fluorescence intensity. The resulting dependence of the normalized specific fluorescence signal ($I_{Eu}/I_{IS}$) *versus* the PBA concentration is presented in Figure 4b. The parameters of the sigmoidal fit of the competitive immunoassay are: $IC_{50}$ = 14 µg $L^{-1}$, slope = -0.6, $R^2$ = 0.987. The detection limit defined as 20% inhibition corresponds to 1.4 µg $L^{-1}$ PBA which is comparable with the reported ELISA for the same analyte *(37)*. This work suggests the potential application of lanthanide oxide nanoparticles as fluorescent probes in microarray and biosensor technology, immunodiagnostics and high-throughput screening.

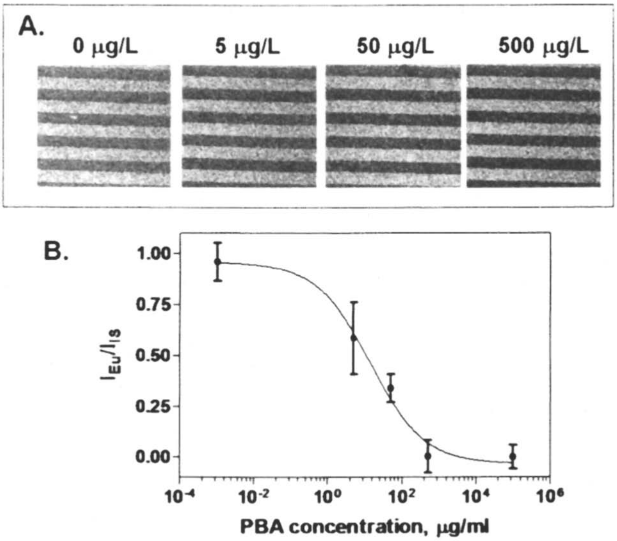

*Figure 4. Microimmunoassay for 3-PBA. (A) Fluorescence images of glass substrates incubated with different concentrations of 3-PBA. (B) Standard curve for the PBA microimmunoassay. (with permission from Nichkova, 2005) (See page 1 of color inserts.)*

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## Outlook

Our laboratory continues to explore new immunoassay approaches such as a non-labeled immunosensor based on ellipsometry *(74)*. Ellipsometry measures the molecular density of thin films. When the antibodies leave the surface, ellipsometry detects the change in molecular density. Larger antibodies or antibody complexed with other proteins could be used to increase the signal.

The optical properties of colloidal crystals provide a method of transducing biological binding to optical signals. Optical signals are fast and easily used in electronic devices. When assembled, these colloids refract light at a certain wavelength. When the crystals with an antibody are coated on the substrate, the refracted light will change wavelength when the ligand binds to the antibody. The wavelength shifts are based on changes in size, charge and shape of the colloids *(75)*,

Although there are many demonstrations of antibody-based biosensor technology, there are few examples of application to real world environmental samples for pesticides *(76)*. New approaches as well applying what we have learned to develop new systems such as a simultaneous multiplex assay using the flow cytometric system with inorganic lanthanide labels instead of the classic organic fluorophores we hope will lead to systems well-suited for human biomonitoring as well as environmental monitoring, making immunoassays even more valuable tools.

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## Chapter 10

# Recombinant Antibodies for Agrochemicals: Evolutionary Optimization

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Genetic engineering of recombinant antibodies (rAb) enables the selection and improvement of fundamental Ab properties such as affinity or thermal stability by rational and evolutionary design. Different ab gene sources are available for this purpose. The human combinatorial Ab library HuCAL® was applied as an example of a fully synthetic Ab repertoire. Specific clones were selected from the HuCAL® library by means of phage display for the detection of the herbicide glyphosate, as well as for food-borne pathogens. Depending on the germline Ab gene family individual Ab fragments exhibited extraordinarily high stability. As an example for a natural rAb library, murine Ab genes were cloned from immunoselected, *s*-triazine stimulated B cell repertoires. The most efficient strategy for the affinity improvement of rAbs for atrazine was found to be a combination of randomized point mutations and sequential recombination of variable domain encoding Ab gene repertoires. This strategy resulted in a shift of the equilibrium dissociation constant $K_D$ from $1.3 \times 10^{-8}$ M of the template Ab to $7.5 \times 10^{-10}$ M of the optimized variant. The applicability of the optimized Ab variant was confirmed by the determination of atrazine contamination in soil samples.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Sensitivity and selectivity of bioanalytical assays for monitoring agrochemicals essentially depend on the properties of the biorecognition elements to be used for analyte binding. These include enzymes, antibodies, hormone receptors, DNA, membrane components and even organelles or entire cells. Immunochemical assay systems employing Abs as binding proteins are effective tools for the analysis of a wide variety of analytes ranging from low molecular weight agrochemicals (e.g. herbicides, insecticides) to complex proteins (e.g. structures of pathogenic microorganisms). Many environmental applications of Abs have been reported in the area of biosensors [for recent reviews see Sharpe (*1*), Paitan *et al.* (*2*), Rodriguez-Mozaz *et al.* (*3*)].

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## Advantages of the Recombinant Antibody Approach

Compared with conventional Abs obtained by hybridoma technology recombinant Ab (rAb) technology facilitates a faster and cheaper production, if the technique and cell line is already established in the laboratory. Whereas the hybridoma technology is predominantly restricted to mammalian cells, rAb production offers a wide choice of different host organisms as expression systems. Even more important is the possibility to optimize analytical properties such as affinity, selectivity towards defined targets, and stability. Furthermore, the structure of rAbs can be adapted to various purposes, such as directed immobilization on sensing surfaces or construction of fusion proteins. Since recombinant Ab sequences can be easily modified by *in vitro* methods, synthetic sequence motifs can be introduced, which are not occurring in natural repertoires. Finally, once a library is established, it can be extended *in vitro* by variation or combination with other libraries. Alternatively, naïve Ab libraries are accessible, from which new Ab selectivities can be directly isolated without any donor passage. These Abs can be used as a template structure for further optimization *in vitro*.

## Existing rAbs for Agrochemicals

An increasing number of research groups utilizes rAb technology for the detection of agrochemicals. When rAb production entered this area, the main strategy consisted of the direct cloning and functional expression of Ab encoding genes derived from hybridoma cell lines. This is mainly due to the fact that groups, which are engaged in Ab production, frequently have access to hybridoma cell lines. Since hybridoma cells are secreting monoclonal Abs of defined analytical characteristics, the success or failure of a cloning experiment can be conveniently validated by comparing the analytical properties of parental monoclonal Abs and their recombinant derivatives.

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The first cloning experiments in the agrochemical area were described at the beginning of the nineties (*4*). These pioneering attempts still encountered a multitude of technical challenges. Thereafter, recombinant Ab fragments were synthesized from hybridoma cells against a panel of relevant agrochemicals and industrial contaminants like diuron (*5*), paraquat (*6*), atrazine (*7*), cyclohexanedione (*8*), parathion (*9*), dioxin (*10*), picloram (*11*), mecoprop (*12*), chlorpyrifos (*13*), coplanar polychlorinated biphenyls (*14*), and others.

Like the parental monoclonal Abs these rAb fragments were appropriate for the quantitative detection of xenobiotics. They were either similar to the parental monoclonal Ab (*10,13*) or they showed altered analytical characteristics (*6,7*). Changes in the binding characteristics compared to the parental Ab, which is secreted by a hybridoma cell, can be essentially traced back to three different reasons.

1. Changes of the Ab design. In addition to a reduction in valency from bi- or polyvalent Abs of the hybridoma cells to monovalent rAbs, the structural association of the heavy and light chain moiety may be affected by genetic engineering. For instance conformational changes may be introduced by artificial linkers, which are frequently used for the stabilization of the rAb heterodimer.
2. Restricted nucleotide sequence alterations may arise as a consequence of the cloning process. These include point mutations, nucleotide insertions or deletions as a result of PCR artifacts. Furthermore, the ligation with the expression vector may evoke additional frameshifts resulting in altered amino acid coding.
3. One or both of the cloned Ab genes may be derived from the myeloma cell partner rather than from the genome of the immortalized B-lymphocyte.

Only recently an increasing number of Ab libraries is successfully employed to select suitable Ab fragments for the detection of agrochemicals from larger Ab repertoires. The corresponding ab genes were isolated from various donor species such as sheep (*15*), rabbit (*16*) llama (*17*), mouse (*18*) and others.

## Recombinant Ab Formats and Fusion Proteins

Ab fragments prepared for environmental analysis are predominantly produced either in the scFv (*8,9*) or $F_{ab}$ format (*4,19*). $F_{ab}$ fragments are comprised of the entire Ab light chain and the corresponding part of the Ab heavy chain. The scFv fragments are truncated to the variable domains of the Ab heavy and light chain, which are connected by an artificial peptide linker. Ab fragments that are fused with marker proteins offer the advantage of a reduced number of assay steps in the analytical test format. The genetically engineered fusion of the Ab binding function with marker enzymes was already reported for environmental analysis (*20*). The corresponding vector contained an alkaline phosphatase and generic restriction sites for the convenient one-step cloning of scFv fragments isolated from Ab library repertoires. Similarly, a gene encoding green fluorescent protein was inserted into a vector harboring a picloram-specific Ab fragment (*21*). The resulting "fluobody" avoids the enzyme-substrate reaction for calorimetric detection that is required in a conventional ELISA.

## Optimization Strategies for rAb Properties

The alteration of the affinity profile of existing Ab by genetic engineering is considered a powerful instrument to circumvent classical Ab production schemes, which require extended immunization periods (*22*). Although this point is frequently raised in environmental research, the issue has yet hardly been translated to successful experimental results. In the environmental area the optimization of Ab fragments *in vitro* was addressed by both, directed evolution (*23,24*) and rational design (*25-27*). In the latter case a triple mutant $F_{ab}$ fragment was generated with an affinity 5-fold higher towards an atrazine derivative as compared to the wild-type (*27*).

### Variation and Selection as Basic Steps of the Evolutionary Strategy

Essential steps of the strategy are depicted in Figure 1. The first step involves the generation of a repertoire of Ab variants, which provides a high level of functional diversity. This repertoire is subsequently expressed in appropriate systems, such as phage display (*28,29*), cellular display on the surface of bacteria or yeast (*30,31*), ribosome display (*32*), and mRNA display (*33*). Then the diverse repertoire is exposed to a selection pressure. For instance an improved level of affinity to the ligand is required for the protein variant in order to be selected. This step corresponds to the survival of individuals in a population of organisms under natural selection pressure. Following selection the best variants are identified by characterizing the ligand-binding properties. If the selected variants do not meet the required analytical properties, one or several variants are used as templates for subsequent molecular diversification in order to generate a new protein repertoire, which can be selected again. This evolutionary strategy might necessitate several rounds of variation and selection until the required properties are achieved.

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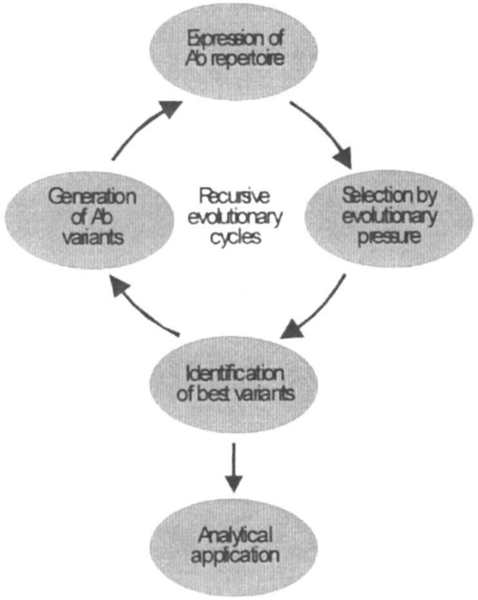
Expression of Ab repertoire
Selection by evolutionary pressure
Identification of best variants
Generation of Ab variants
Recursive evolutionary cycles
Analytical application

*Figure 1. Principle of directed evolution*

## Synthetic and Natural Recombinant Antibody Libraries

Two fundamentally different approaches can be distinguished for the generation of molecular diversity: The generation of synthetic repertoires and the cloning of natural repertoires by *in vitro* methods. Whereas the latter method is based on naturally occurring genes, the first strategy generates Ab sequences, which do not necessarily exist in nature.

In a first approach, we employed the fully synthetic human combinatorial Ab library HuCAL® (MorphoSys, Inc., Martinsried, Germany) for the selection of Ab variants. The library is based on consensus sequences for each of those seven variable heavy chain ($V_H$) and variable light chain ($V_L$) germline families, which are most frequently used in the human immune response. Diversity is created by replacing the $V_H$ and $V_L$ complementarity-determining regions CDR3 of the master genes by CDR3 library cassettes, generated from mixed trinucleotides (*34*) and biased towards natural human Ab CDR3 sequences (*35*).

Specific clones were selected from the HuCAL® library by means of phage display for the detection of the herbicide glyphosate (N-(phosphonomethyl)glycine) and several food-born pathogens. The stability of selected Ab fragments was investigated by incubation at varying concentrations

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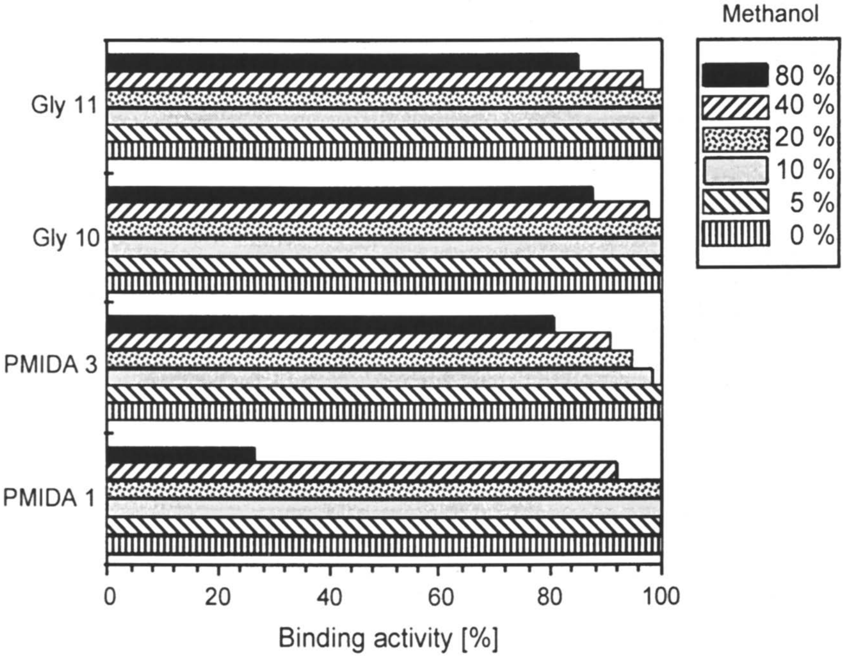

*Figure 2. Methanol sensitivity of glyphosate-selective Ab clones isolated from HuCAL® library. Binding activity was evaluated by measuring the maximum and minimum signal in ELISA with sample buffer containing 0-80% (v/v) methanol (MetOH) according to the formula: ($A_{max}$ - $A_{min}$ at x% MetOH)/ ($A_{max}$ - $A_{min}$ at 0% MetOH) x 100%.*

of methanol. Functionality was subsequently investigated by ELISA. A content of 40% (v/v) methanol in the sample did not significantly affect the binding potential of the majority of the glyphosate-selective Ab fragments (Figure 2) (*36*).

Even a content of 80% (v/v) methanol showed a relevant signal reduction in ELISA just for one out of the four tested Ab clones. This is noteworthy since in similar investigations based on conventionally produced polyclonal and monoclonal Abs we observed that the majority of Abs lost their binding capability at much lower concentrations in the range between 5 to 15 % (v/v) organic solvent (*36*).

The thermal stability of the Ab fragments can be used as an indicator for their long-term storage properties and molecular integrity at unfavourable temperature conditions (e.g. no cooling device available during outdoor tests). In order to investigate this issue, Ab fragments were incubated for 24 h at various temperatures and their functionality was tested by ELISA again. The corresponding results for the pathogen-selective Abs are presented in Figure 3 (*36*). Almost all Ab fragments were stable up to temperatures of 37 °C and

50 °C, respectively. Most of the Abs showed reduced or even completely lost ligand binding at higher temperatures. These observations are consistent with analogous experiments performed with conventional monoclonal Abs derived from hybridoma cultures in our laboratory. However, one out of the six fragments depicted in Figure 3 retained full functionality even after incubation at 80 °C, which indicates an exceptional temperature stability.

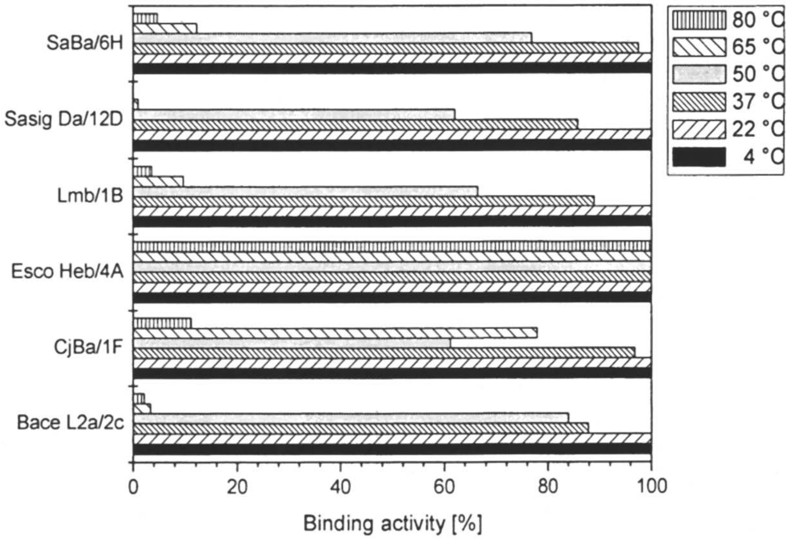

*Figure 3: Thermal stability of pathogen-selective Ab clones isolated from the HuCAL® library. Ab fragments were isolated for the detection of Staphylococcus aureus (SaBa), Salmonella spec. (Sasig), Listeria monocytogenes (Lmb), Escherichia coli (Esco Heb), Campylobacter spec. (CjBa) and Bacillus cereus (Bace L2a). Ab fragments were incubated for 24 h at temperatures ranging from 4-80 °C. Binding activity was subsequently evaluated by measuring the maximum and minimum signal in ELISA according to the formula: ($A_{max}$ - $A_{min}$ temperature treated Ab)/($A_{max}$ - $A_{min}$ of untreated control) x 100%.*

Sequence alignment with human germline genes revealed that the immunoglobulin variable heavy chain genes of the clones providing the highest stabilities could be predominantly assigned to the $V_H3$ gene family. This is at least a hint that the stability of Ab fragments may be an inherent structural feature of particular germline families. These results are well in line with investigations of the biophysical properties of Ab fragments performed by other groups employing the HuCAL® library (*37*). Ab fragments containing the

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variable domain combinations H3κ3 and H5κ3 showed superior stability. Combination with λ light chains also exhibited high levels of stability depending on the particular amino acid sequence of the CDR-L3.

An entirely different approach to generate Ab diversity is the cloning of Ab gene repertoires, which are isolated from donor organisms. The corresponding repertoire is expected to be unbiased, which means that the immune system of the donor organism has not been challenged by an antigen. These naïve repertoires theoretically harbor Abs for any target structure, however at a moderate affinity level for the majority of binding molecules. In contrast, biased Ab repertoires can be cloned from immunized sources. The latter strategy benefits from *in vivo* mechanisms of the immune system, since Ab variable genes encoding the antigen binding domains are modified during the secondary immune response in the microenvironment of lymphoid germinal centers by somatic hypermutation. Appropriate variants are subsequently selected from this pool of mutant immunoglobulins upon their improved affinity to the antigen (*38*). Therefore, immunizing an organism with a specific antigen serves as an *in vivo* preselection of potent Ab genes.

We developed a strategy to generate a group-selective library representing a large natural Ab gene pool. This library was designed to include a range of *s*-triazine-specific Abs in order to facilitate the selection of Abs against defined members of the *s*-triazine family (*23*). For this purpose, Ab-secreting B cells were derived from Balb/c mice, which had previously been immunized with different *s*-triazine immunogens including derivatives of atrazine, ametryn, terbuthylazine, deethylatrazine and simazine. B cells secreting *s*-triazine-selective Abs were separated from unspecific B cells by means of immunomagnetic separation. This method takes advantage of the membrane-bound receptor molecules on the B cell surface, i.e. transmembrane protein complexes that share their ligand-binding characteristics with the secreted Ab. These surface receptors can be tagged by target molecules covalently linked to paramagnetic particles. After exposing the particles to a magnetic field, specific B cells were removed from bulk cultures by magnetic force (*39*). Ab-encoding genes from magnet-bound B cells were subsequently cloned into a phage display system. The resulting library comprised all target relevant sequences that have been included in the starting B cell repertoire.

This group-selective Ab library was then used to select Ab variants selective for s-triazines containing a tertiary butyl group (BUT), i.e. terbutryn and terbuthylazine as well as those s-triazines bearing an isopropylamino residue (IPR), i.e. atrazine and propazine. Specific phages were enriched by three repetitive cycles of selection applying immunoaffinity chromatography with BUT derivative-coated and IPR derivative-coated columns.

Binding studies for the characterization of selected Ab clones were essentially performed by an enzyme-linked immunosorbent assay (ELISA) and an optical biosensor. The clones could be selectively displaced by atrazine as

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indicated by the corresponding calibration curves (Figure 4). Reaction kinetics of the three best binding clones for triazine derivatives containing tertiary butylamino and isopropylamino groups were determined with the BIAcore 2000 sensor. The binding constants $K_D$ ranged from 1.2 x $10^{-8}$ M for IPR-7 to 8.0 x $10^{-8}$ M for BUT-56, which corresponds to the affinity level of maturated Abs that are obtained during the secondary immune response (23).

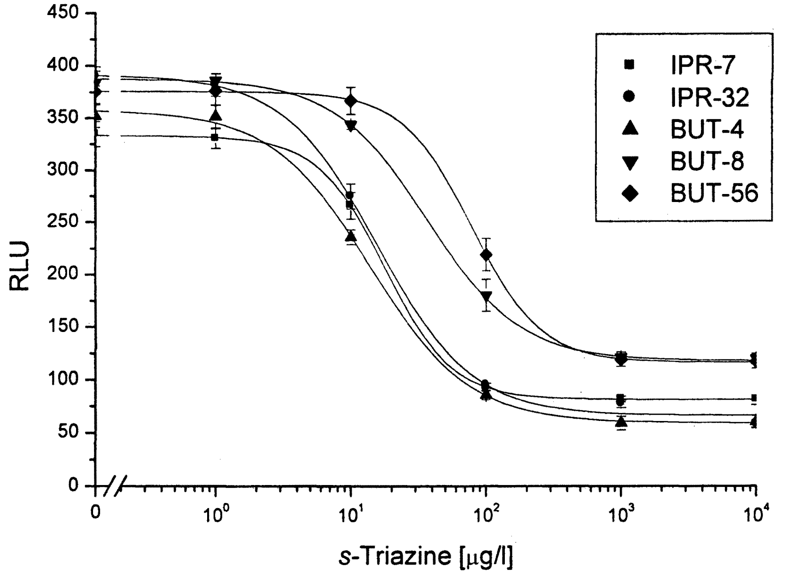

*Figure 4. Calibration curves of IPR and BUT clones for the analysis of atrazine and terbutryn, respectively, by direct heterogeneous competitive ELISA. IPR and BUT clones were selected by phage display technology employing s-triazine derivatives, which contain isopropylamino (IPR) and butylamino (BUT) residues. RLU: Relative luminescence units.*

## Affinity Optimization of Atrazine rAbs

Once established, primary synthetic or natural Ab libraries can be subsequently subjected to sequence diversification *in vitro* in order to enhance the immune repertoire (c.f. below). Frequently applied *in vitro* methods include random and directed nucleotide alterations or recombination techniques (*40*). Random techniques include the introduction of point mutations by error prone PCR. Nucleotides throughout the entire gene are randomly exchanged (*41*). In

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contrast to random methods, oligonucleotide-directed procedures insert synthetic sequences into the gene of interest at specific sites, e.g. strand overlap extension (*42*) or megaprimer-based PCR (*43*). Recombination techniques are based on randomized recombination of gene segments or complete genes (*40*) (e.g. recombination of Ab $V_H$ and $V_L$ encoding genes).

*In vitro*, Ab genes are subjected to iterative evolutionary cycles of mutation and selection, until they meet the requirements for the designated application (c.f. Figure 1). The gene repertoire of the group-selective Ab library presented above was employed for subsequent optimization of individual Ab molecules by directed evolution (*44*). It was expected that this library would substantially facilitate the engineering of desired Ab specificities and affinities to any member of the triazine group without the need of new immunizations.

The Ab clone IPR-7 was used as a template for the optimization process. This clone was initially selected from the group-selective library (c.f. Figure 4) and provided the highest affinity to *s*-triazines bearing an isopropylamino residue, i.e. atrazine and propazine (Table I) (*44*). Chain shuffling was applied as a directed, recombinatorial approach for improving the affinity of this clone. At the outset the light chain of the template Ab IPR-7 was shuffled with the heavy chain repertoire of the group-specific library, and subsequently selected by phage display. Then, the heavy chain of the best binder (IPR-26) was shuffled against the library light chain repertoire. The kinetic data of the Ab variants are detailed in Table I.

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**Table I. Kinetic Characterization of scFv Clones**

| Clone | $k_a$ ($M^{-1}s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D = k_d / k_a$ (M) |
|---|---|---|---|
| IPR-7 | $1.38 \times 10^5$ | $1.75 \times 10^{-3}$ | $1.27 \times 10^{-8}$ |
| IPR-26 | $2.10 \times 10^5$ | $1.93 \times 10^{-3}$ | $9.20 \times 10^{-9}$ |
| IPR-83 | $6.73 \times 10^5$ | $5.02 \times 10^{-4}$ | $7.46 \times 10^{-10}$ |

NOTE: Association rate constant $k_a$, dissociation rate constant $k_d$ and equilibrium dissociation constant $K_D$ for IPR. The values for $k_a$, $k_d$ and $K_D$ were measured with immobilized IPR derivative utilizing the BIAcore 2000™ system.

The equilibrium dissociation constants of the Ab variants are approaching the typical $K_D$ level of affinity matured Ab *in vivo* (*45*). The optimized variant IPR-83 showed a 17-fold increase in affinity as compared to the the template Ab IPR-7. Interestingly, sequence analysis of the shuffled clones revealed a bias of amino acid substitutions from the template IPR-7 to the optimized variant IPR-83 in the 5' moiety of the V genes including the first two CDRs and the flanking frame regions (*44*). This is in contrast to a series of Ab optimization experiments that are primarily targeting on the variation of the CDR3 regions at the 3' moiety of the Ab gene (*22,25*). The $V_H$ CDR3 region is generally considered to constitute the key determinant for the antigen selectivity (*46*). However, the distribution of sequence alterations obtained in the presented Ab optimization is consistent with proposed models for mutational mechanisms during the secondary immune response *in vivo* (*47,48*). In addition, experimental data obtained from *in vivo* immune repertoires are confirming individual sites at the V genes that are prone to hypermutation. These mutational "hot-spots" for affinity maturation are strategically located at the CDR1 and CDR2 rather than in the CDR3 loop (*49,50*). Therefore, the applied *in vitro* optimization strategy resulted in a distribution of sequence alterations, which is fitting very well into the current knowledge of natural affinity maturation.

The applicability of the optimized Ab variant IPR-83 was tested by measuring environmental samples. IPR-83 was applied to determine atrazine contaminations of soil samples collected in Southern Germany. Although atrazine was banned in Germany by the European Community in 1991, environmental contaminations have been observed during the last years due to illegal applications. The corresponding threshold for atrazine is 100 μg/kg soil. The immunochemical analysis was complemented by HPLC measurements as reference method for validation (Table II). The ELISA data were consistent with the HPLC measurements within the experimental error (*44*). Thus, the engineered $scF_v$ mutants proved to be suitable for the application in environmental analyses under real sample conditions.

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## Discussion and Outlook

The principles of directed evolution *in vitro* are very similar to the mechanisms of somatic mutation *in vivo*. The Ab repertoire of the primary immune response *in vivo* is predominantly the result of recombining germ line genes of the V, D, J clusters (i.e. the genes constituting elements of the finally expressed $V_H$ and $V_L$ genes). The primary response yields Abs with generally low affinities. Thereafter, Ab variable genes of the primary repertoire are modified during the secondary immune response in the microenvironment of lymphoid germinal centers by somatic hypermutation. Physiologically appropriate variants are subsequently selected from this pool of mutant immunoglobulins upon their improved affinity to the antigen (*38*).

The primary repertoire *in vitro* is established by cloning synthetic or natural Ab genes as described above for the fully synthetic HuCAL® and the group-selective library, respectively. The former one is a commercially available library. Other examples of accessible primary repertoires include the Tomlinson

**Table II. Atrazine determination of soil samples.**

| *Soil sample* | *ELISA atrazine [μg/kg]* | *HPLC atrazine [μg/kg]* |
|---|---|---|
| 1 | 43 ± 4 | 49 ± 3 |
| 2 | 323 ± 8 | 313 ± 12 |
| 3 | 35 ± 8 | 33 ± 3 |
| 4 | 91 ± 13 | 85 ± 10 |
| 5 | 126 ± 4 | 120 ± 21 |
| 6 | 141 ± 6 | 135 ± 14 |
| 7 | 65 ± 5 | 59 ± 8 |
| 8 | 108 ± 11 | 115 ± 9 |
| 9 | 121 ± 3 | 113 ± 7 |

NOTE: Soil samples were measured by ELISA employing the mutant antibody IPR-83 (c.f. Table 1). Validation was performed by HPLC. HPLC data were kindly provided by Dr. J. Lepschy, Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising, Germany.

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I and J Ab libraries (*51*). Some of these primary libraries are very large (containing more than $10^{10}$ different Ab variants) and therefore considered to be 'universal', which suggests that the corresponding Abs are covering a huge panel of various specificities. However, even at its best these libraries will represent no more than a basic immune repertoire. The optimization by means of molecular evolution strategies or alternatively by rational design probably remains therefore an integral part in the synthesis of appropriate Abs for the majority of immunochemical applications in environmental analysis.

Therefore, one of the vital goals for the future is the simple and cheap access to evolutionary technologies for tailored binders with predefined properties such as selectivity, affinity, stability and more. Massive parallel processing combined with high-throughput strategies as well as high-content screening will have a beneficial impact on this new era of Ab production. The hardware is already available for a straightforward production of rAbs. From automated plating and picking of bacteria colonies up to robotic screening and data processing. However, self-controlled variation and selection has to be integrated in the concept too. For instance, recombination *in vivo* can be achieved by coupling chain-shuffling with the phage infection of bacteria as demonstrated for the cre/lox system. An Ab light chain repertoire in the phage is recombined with the heavy chain repertoire in the bacteria host by infection (*52*). Selection of specific Ab variants can be controlled by integrating the selectively infective phage (SIP) technology (*53*). Recombinant phages displaying the Ab fragments on their surface are lacking the protein domain, which enables the infection of bacteria. However, infectivity can be restored by selective binding of the displayed Ab to the ligand, which is coupled to the infective protein domain.

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Cell free systems like ribosome and RNA display paved the way for larger ab repertoires *in vitro* and an accelerated variation and selection cycle. Ribosome display (*32*) has been already successfully applied for the production of agrochemical selective rAb (*17*). However, the non-covalent complexes of ribosome display are relatively unstable and may dissolve during stringent selection conditions. RNA display is considered as a major technical improvement in this respect. Here the encoded protein is covalently linked to the mRNA and therefore less susceptible to dissociation than the ternary ribosome complex (33).

Another promising area is the exploitation of the potential of alternative Ab molecules. For instance, single domain Ab (sdAb) are naturally occurring in camelids and sharks (*17,54,55*). In addition to Ab domains, so-called Ab mimics have been reported to be amenable to molecular optimization. The tenth fibronectin type III domain ($_{10}$Fn3) (*56*), a monomeric member of the immunoglobulin superfamily, and the extracellular domain of human cytotoxic T-lymphocyte associated antigen (CTLA4) (*57*) were used as a scaffold for library synthesis. In addition to immunoglobulins and immunoglobulin-like polypeptides, some specific affinity reagents have been selected from small, globular protein scaffolds not related to Ab. For instance, the α-helical Z-domain of protein A, designated as "affibodies" was improved in directed evolution experiments to the nanomolar range (*58*).

Similarly, the bilin-binding protein (BBP), a lipocalin from the butterfly *Pieris brassicae*, was used for library synthesis (*59*). The protein has a conserved β-barrel core formed by eight antiparallel β-strands. Peptide loops, which connect the individual strands, confer binding to the ligand. Affinities in the picomolar range for digoxigenin were achieved by selective mutation of these "anticalins" (*60*). Another type of protein scaffold is the ankyrin repeat (AR). AR proteins are composed of several 33 amino acid repeats stacked in a row. Each repeat comprises a β-turn followed by two antiparallel α-helices and a C-terminal loop reaching the β-turn of the adjacent loop (*61*). Synthetic libraries were developed by randomizing amino acid positions at the β-turn and the short hinge connecting the two α-helices (*62*). The AR libraries yielded high-affinity binding variants with $K_D$ in the nanomolar range against various protein targets. These binding proteins show that there are promising candidates with a potential for environmental analysis, which could provide an alternative for the classical Ab molecule.

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## Chapter 11

# Noncompetitive Fluorescent Immunoassay for the Detection of the Human Urinary Biomarker 3-Phenoxybenzoic Acid with Bench Top Immunosensor KinExA$^{TM}$ 3000

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A sensitive, automated, non-competitive fluorescent immunoassay was developed for quantitative analysis of 3-phenoxybenzoic acid (PBA) in human urine sampes as a putative biomarker of exposure to pyrethroid insecticides using the bench top immunoanalyzer, KinExA$^{TM}$ 3000 system. The key difference between the KinExA system and the enzyme-linked immunosorbent assay (ELISA) is to eliminate the PBA-antibody interaction with the coating antigen. This can be achieved by separately capturing the free PBA-antibody onto the hapten-immobilized beads when a constant amount of reaction solution in equilibrium between PBA-antibody and analyte passes through the bead-packed glass capillary column. Optimal dilution of the PBA antibody was determined when fluorescent signals of 0.5-2 were obtained and a sufficient amount of coating antigen was immobilized to ensure the capture of all free antibodies. $IC_{50}$s of the two KinExA methods (0.3 and 0.6 ng/mL for one- and two-step KinExA, respectively) were 3- and 6-fold better than the heterologous ELISA and were approximately 650- and 300-fold lower compared to that of the homologous ELISA ($IC_{50}$ of 200

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

ng/mL). The KinExA assay was negligibly affected within tested range of pHs (5-10) and ionic strengths (1, 5, and 10X PBS). Similar urine matrix effects were observed in the two KinExA assays with a 5- to 10-fold increase in $IC_{50}$s when 5 and 10% of urine was contained in the reaction buffer. A high correlation ($r^2$ = 0.99) was observed between detected and spiked concentrations of PBA standard with average recoveries of 88-160%.

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## Introduction

Immunoassay has proven to be a sensitive tool to detect environmentally relevant substances such as pesticides or other toxic compounds in a variety of sample matrices (*1, 2*). The major merit of an immunoassay is the antibody-driven, high selectivity and sensitivity, which enables one to simplify sample preparation leading to rapid analysis of samples and to perform high-throughput analysis with very small sample volume. For immunoassay development, monoclonal antibodies (MAb) have some advantages over polyclonal antibodies (PAb) and are preferable to many researchers, however, PAb also gives comparable selectivity and sensitivity for immunoassay development particularly for the detection of small molecules. Enzyme-linked immunosorbent assay (ELISA) is the most widely accepted immunoassay format reported to date. ELISA methods are divided into two major formats, non-competitive and competitive. Non-competitive ELISA is mostly applicable to the detection of macromolecules such as proteins with at least two antibody binding sites. However, for the detection of small molecules, this type of non-competitive ELISA is not applicable because once the molecule binds to antibody; there is no site available for the binding of reporter molecules. So competitive ELISA is an alternative to non-competitive ELISA for small molecules. Although competitive ELISAs provide satisfactory sensitivities they are limited by the equilibrium between primary antibody and coating antigen. In other words, when antibodies have a higher affinity for the coating antigen than to the analyte during the competition step, high concentrations of analyte must be added to inhibit antibody binding to the coating antigen. This results in increased $IC_{50}$ values. Thus, for the development of a sensitive competitive ELISA for a small molecule, it is essential to synthesize a series of competing haptens that have minor structural modifications of the immunizing hapten. These haptens must be screened to find one with lower affinity to the antibody than the target compound (*3-5*). The synthesis of competing haptens involves the use of hazardous chemicals and time consuming and laborious procedures. There have been efforts to develop non-competitive immunoassays for small molecules. Two types of non-competitive immunoassays have been reported. The first is to use anti-idiotype antibodies generated by injecting antibody-analyte complex or recombinant antibody by recombinant DNA techniques (*6-8*). With these types of antibodies, assays can be conducted in a 96-well plate in a format similar to the sandwich-type ELISA. The second is to remove antibody interaction with the coating antigen by separating the interaction with analyte from the coating antigen so that assay sensitivity is solely dependent on affinity of antibody to target compound (*9,10*). For this, the antibody is first allowed to reach equilibrium binding with the analyte, then free antibody is separated from antibody-analyte complex. Detection can be conducted without elution or after elution of captured free antibody. Eluted antibody-analyte complex also can be used for quantification. These methods do not require synthesis of competing haptens. However, they are somewhat complicated, necessitating repeated capture and elution of antibodies for separation and quantification. To further simplify assay procedures and improve assay sensitivity, we report a very sensitive automated non-competitive flow fluorescent immunoassay for the detection of PBA in human urine samples. The PBA PAb used is one that showed a 100-fold difference in $IC_{50}$s between heterologous and homologous coating antigens in the ELISA.

Pyrethroids act on the axons of the nervous system by interacting with sodium channels in insects and mammals (*11*). The properties of pyrethroids such as high potency in controlling a wide spectrum of insects and low toxicity to birds and mammals have made it accepted worldwide for application in agriculture, forestry, homes, horticulture, and public health (*12-14*). Although pyrethroids are considered safe for humans, there have been concerns about long-term low level and high exposures, as well as environmental accumulation, and leaching into surface and groundwater (*15,16*). Some research has revealed that humans exposed to high levels of pyrethroids may experience suppressive effects on the immune system, endocrine disruption, lymph node and splenic damage, and carcinogenesis (*17-19*). Pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage mainly to *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA) and PBA, which are further processed to conjugates such as glucuronide, glycine, taurine, and sulfate (*20,21*). Therefore, the development of a sensitive immunoassay for PBA may be a useful tool to estimate human exposure to pyrethroid insecticides. We have reported several immunoassays for the detection of pyrethroid parent compounds (*22-26*), their primary and secondary metabolites (*27-29*).

In this study, we used the Kinetic Exclusion Assay system (Sapidyne Instruments, Boise, ID), a bench top immunoanalyzer, to develop a non-competitive immunoassay for PBA detection. Limited use of the KinExA has been explored for quantitative analysis (*30-33*). In this paper, we compared two

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KinExAs to heterologous and homologous ELISAs, and used the KinExA assay to analyze PBA in human urine samples.

## Materials and Methods

### Reagents

All reagents were of analytical grade unless specified otherwise. Bovine serum albumin (BSA), goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP), 3,3',5,5'-tetramethylbenzidine (TMB), all chemicals for buffer preparation, and PBA were from Sigma (St. Louis, MO, USA). Goat anti-rabbit IgG conjugated with Cy5 (GAR-Cy5) and Cy5 conjugation kit were purchased from Amersham Bioscience (Piscataway, NJ, USA). Desalting columns and the protein A affinity purification kit was purchased from Pierce (Rockford, IL, USA). Polymethylmethacrylate (PMMA) beads were purchased from Sapidyne (Boise, ID, USA). Buffers for ELISA and KinExA assays were normal strength PBS [1X PBS; 8 g/L of sodium chloride (NaCl), 0.2 g/L of sodium phosphate dibasic anhydrous ($Na_2HPO_4$), and 0.2 g/L of potassium chloride (KCl), pH 7.5], PBST (PBS containing 0.05% Tween 20), carbonate buffer [1.59 g/L sodium carbonate ($Na_2CO_3$), 2.93 g/L sodium hydrogen carbonate ($NaHCO_3$), pH9.6], and 0.05M citrate-acetate buffer (14.71 g/L $Na_3C_6H_5O_7{\cdot}2H_2O$, pH 5.2). The structures of PBA and its immunizing and coating haptens are shown in Figure 2. Synthesis of the haptens for the production of PAb and assay development were described in our previous paper (*29*).

### Immobilization of PBA Immunizing Hapten-BSA Conjugate to PMMA Beads

Dry PMMA beads (200 mg, 98-µm diameter) in a 1.5 mL Eppendorf tube were suspended in 1 mL of nanopure water and washed two times with PBS buffer by centrifugation and removal of the supernatant solution. Beads were then suspended with 1 mL of coating buffer, 0.1 mg of PBA hapten-BSA conjugate added, and the tubes were rolled with an end-over-end rocker for 1 h at 37 °C. After discarding the coating buffer, 1 mL of blocking buffer (1% BSA in PBS with 0.05% Tween 20) was added and the tubes rolled again for 2 h at room temperature. After the blocking buffer was discarded, 1 mL of PBS was added. Bead preparations were stored at 4 °C until use. On the day of use, the contents of two tubes were transferred into the bead reservoir of the KinExA along with 27 mL of PBST to provide a constant supply of beads into the capillary flow column.

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## KinExA Principles and Non-competitive Assays

KinExA is an automated flow fluorescent immunoassay system initially intended to determine the true liquid-phase equilibrium dissociation constants ($K_d$) and association rate constants ($k_{on}$) of antibodies by measuring the amount of antibodies in an equilibrium state with ligands in solution phase (*34-36*). The principle is shown in Figure 1 and has been fully described by several laboratories. (*30-36*).

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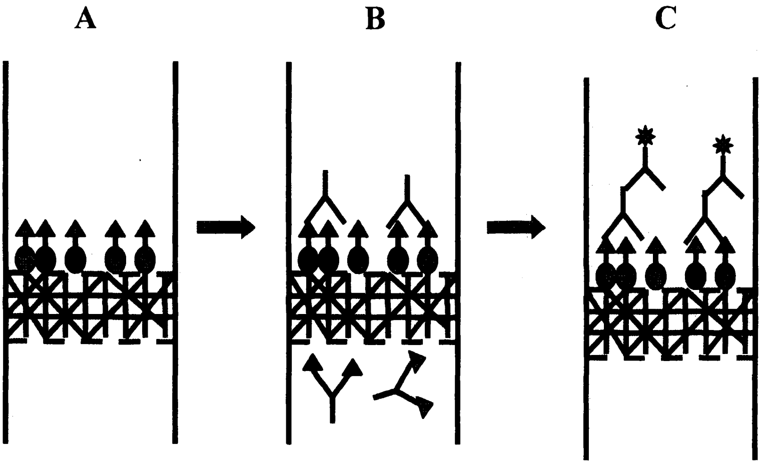

*Figure 1. Schematic presentation of the KinExA principle. (A) Beads are stirred in the bead reservoir for a few seconds to homogeneously disperse hapten-immobilized beads and then transferred onto the microporous screen in the capillary flow cell. (B) Sample from one of the reaction tubes containing a fixed amount of antibody and various concentrations of analyte is injected through the rotary selector into the flow cell. While a sample passes through the cell, free antibody is captured by hapten-coated beads allowing the antibody-analyte complex to flow into the waste reservoir. (C) The secondary antibody-Cy5 is captured onto the bead by the primary PBA antibody. A fluorescent detector reads the signal of captured secondary antibody in the flow cell.*

The resulting fluorescence signal is proportional to the amount of free receptor and inversely to the concentration of analyte. For the one step assay, a constant amount of Cy5-conjugated PBA antibody was mixed with an equal volume of standard solution in various concentrations. Each of 12 injection lines connected to the rotary selector valve was placed into a reaction tube and 13th line into the tube containing 10% NaOH solution. The time for appropriate bead loading in the capillary column was determined by adjusting the time setting parameters of the KinExA software until the height of bead packing matched that of the manufacturer's measuring tool. Four hundred microliters of each sample solution was pre charged into each injection line twice to assure that there were no air bubbles in the lines. The mixtures were then incubated for 30 min at room temperature to allow them to reach equilibrium. Then, 400 µL of each reaction was passed through the bead column. After a wash with PBST, the fluorescent intensity of the captured PBA antibody was obtained. For the two-step assay, the mixture of a fixed amount of unlabeled PBA antibody and each analyte solution in various concentrations was passed through the bead column and the captured antibody was detected by adding GAR-Cy5 (1 µg/ mL). After measuring the signal, the bead column was back flushed and the column was washed successively with PBST and 10% NaOH solution. A new batch of beads was introduced into for the next round of measurement. The standard inhibition curve of twelve different concentrations of PBA was fitted using a four parameter equation with Origin 6.1 software.

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### Microplate ELISA

ELISAs were performed in 96-well microplates as described in Shan et al. (*27*).

### Antibody labeling

Antibody labeling with Cy5 dye was performed following the manufacturer's instruction. The antibody-Cy5 conjugate was separated from free dye with a desalting column. Sodium azide was added to a final concentration of 0.02% and aliquots were stored at -20 °C.

### Effects of Urine Matrices and pH

Assay tolerance to various pH and ionic strengths was estimated using PBS buffer of pH 5, 6, 7, 8, 9, and 10 and 1, 5, and 10X PBS. For an estimation of matrix effect on assay performance, PBS buffer containing 5 and 10% urine was prepared and $IC_{50}$s were compared to that of 0% urine.

### Fortification Urine Samples with 3-PBA

PBA standard stock solution dissolved in DMSO was used for spiking urine samples. Urine samples were spiked with PBA standard and diluted with PBS buffer to render final concentrations of 0.25, 0.5, 5, 10, 15, and 20 ng/mL. The final concentration of urine was kept at 5%.

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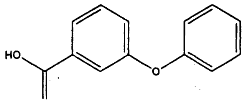
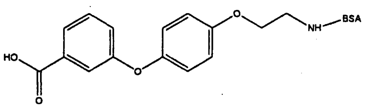
# Results and Discussion

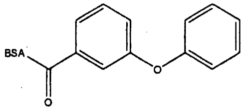
## Non-Competitive Homologous KinExAs and Comparison of Sensitivities with Plate ELISAs



PBA



Homologous coating antigen



Heterologous coating antigen

*Figure 2. Structure of tet compound and coating antigens*

For the homologous non-competitive KinExA, a sufficient amount of coating antigen must be immobilized on the beads to capture all free antibodies. To test this, various dilutions of unlabeled- and Cy 5-labeled antibody were passed through the bead column containing 5 mg of beads on which 2.5 μg of PBA hapten-BSA conjugate was immobilized by adsorption (data not shown). Fluorescent signals measured in real time gradually increased as the amount of antibody increased. Unlike the typical ELISA, it is not necessary to determine the antibody dilution that saturates the immobilized coating antigen. For the two-step KinExA, the highest signal of 8.2 was observed at a dilution of 1:8000. Our previous experience with this system had shown that when the maximal fluorescent signal was set between 0.5 and 2.0, the detector of the KinExA was capable of distinguishing the differences in signals resulting from various concentrations of target compound. Thus, 1:64000 dilution of antibody was selected for the two-step assay, which gave a signal difference of 2. In contrast to the two-step assay, the fluorescent signals were significantly weaker in the one-step. This could be explained by 1) signal amplification due to multiple binding of commercial GAR-Cy5 to the captured PBA antibody in the two-step KinExA, 2) the further dilution of PBA antibody during the Cy5 conjugation and desalting procedures, or 3) probably a low molecular ratio of Cy-5 to antibody. The 1:20000 dilution of PBA antibody was selected for one-step KinExA. At the selected dilution of antibody, most of the free antibody could be captured on the beads. Microplate indirect competitive ELISAs were carried out with homologous and heterologous coating antigens and the sensitivities ($IC_{50}$s) were compared with those of two KinExA assays. In Figure 3, the KinExA grams show the trend in signals in the presence of various concentrations of PBA. In this case, data acquisition was not initiated until the labeled antibody was injected. Since the PBA antibody was not labeled, the two-step assay requires

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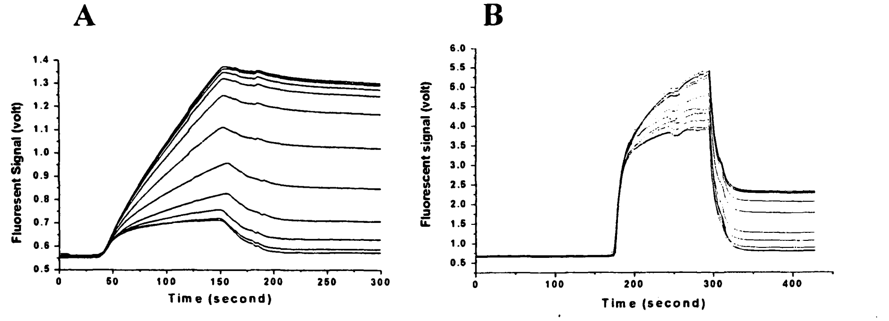

*Figure 3. Example KinExAgrams in the presence of PBA in 0-2500 ng/mL. A. One-step KinExA; B. Two-step KinExA.*

longer initiation times (1-170 s) than the one-step assay (1-25s) for the automatic packing of fresh beads, sample injection, and column wash. There is a substantial signal increase when labeled PBA or secondary antibody is captured on the bead (170-300 and 50-150s for two- and one-step assay, respectively) and the successive washing steps rapidly removed unbound secondary antibody so that established measurable signals were obtained. Signal intensity was inversely proportional to the concentration of PBA. Figure 4 shows representative four-parameter curves by the KinExAs and plate ELISAs. In the microplate ELISA, the $IC_{50}$ of heterologous ELISA (2.0 ng/ml) is 100-fold lower than that of homologous ELISA (200 ng/ml), which indicates that the formation of reverse equilibrium is dominant in solution during

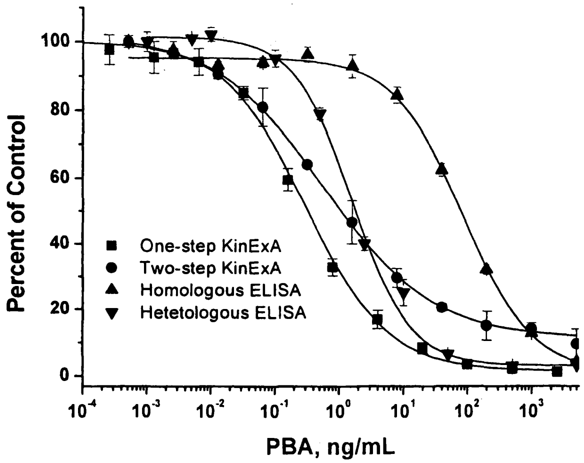

*Figure 4. Four-parameter curves plotted with end-point signals of two KinExAs and ELISAs.*

the competition step. The $IC_{50}$s of the two homologous KinExAs were 0.3 and 0.6 ng/ml for the one- and two-step assay, respectively. Compared to plate ELISAs, a significant enhancement in assay sensitivity was achieved by the homologous KinExA, approximately 650-fold improvement over the homologous ELISA and a 3- to 6-fold improvement over the heterologous ELISA. In the KinExA system, the working range was approximately 0.02-3 and 0.1-20 ng/ml for the one- and two-step assay, respectively. The one-step KinExA assay is more advantageous over the two-step assay because analysis time for each sample is significantly shortened as the addition of secondary antibody is omitted.

This minimizes photobleaching of the fluorescent dye that can cause signal variations from assay to assay. In addition, the one-step assay shows near zero background signals whereas the two-step assay always shows near 20% background signals due to nonspecific binding of secondary antibody. To generate reproducible results, it is important to keep the background signal at a constant level since the final reading signal is the difference between background and endpoint signals. Adsorption of reagents onto the column wall and insufficient washing are a principal cause for a background increase. Beginning the analysis with the most dilute samples and progressing to samples with higher concentrations can also help minimize the problem. Overnight washing of inlet lines with a solution of 0.1 N NaOH can also reduce the background. However for this assay, during assay optimization, the background increased despite the use of these measures. The problem was eliminated by introducing a 10% NaOH solution through the 13th sample injection line immediately after back-flushing the beads. It should be noted that NaOH should not be used throug the h buffer line otherwise

## Matrix Effect

High tolerance of an assay to the changes in pH values and ionic strengths is desirable to accurately detect PBA in human urine samples. Antibody interaction is often affected by the physicochemical properties of the reaction solution. So the effects on the sensitivity of KinExA assay were estimated at various pH values and ionic strengths (Figure 5). For this experiment, PBS buffer with desired pH values and ionic strengths were prepared. This experiment was conducted with the one-step KinExA method. The sensitivity of the assay was negligibly affected within the range of tested pHs of the solutions. Although a slight decrease in maximal signal at pH 4 and 10 was observed, there was no significant difference in $IC_{50}$s indicating that the changes of pH in the range tested would not affect the accuracy. The assay was also highly tolerant to changes in ionic strength with little change in $IC_{50}$s and maximal signals. The effect of urine on assays was also estimated with the two KinExA methods using PBS buffer containing 5 and 10% urine (Figure 6). Effects of urine were similar

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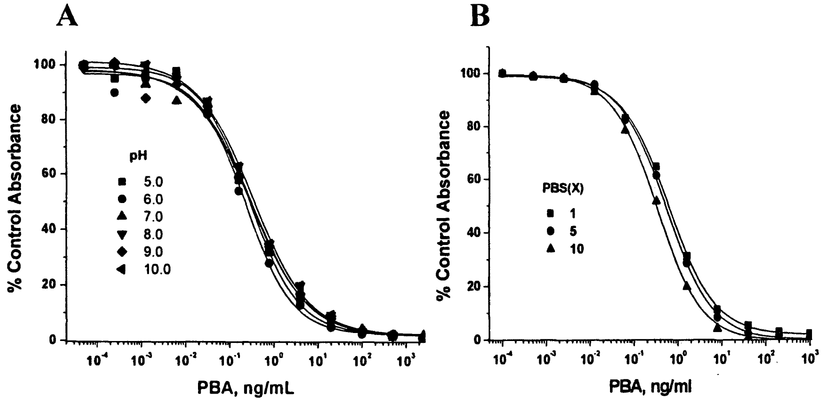

*Figure 5. Effect of various pHs and ionic strengths on assay performance. A. effects of pHs; B. effects of ionic strengths. Each point represents the mean value of two replicates.*

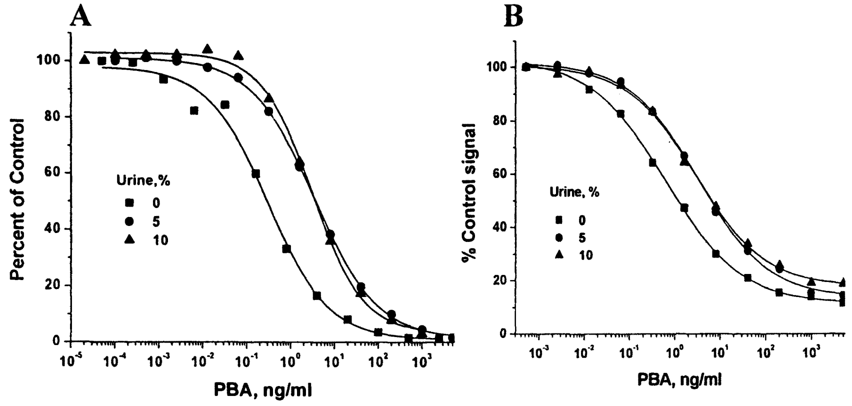

*Figure 6. Effect of urin matrix on assay performance. A.effect of urine on one-step KinExA; B.effect of urine on two-step KinExA. Each point represents the mean value of two replicates*

at the two urine concentrations for the two assay methods. $IC_{50}$ values were 5- to 10-fold increased at 5 and 10% urine. However, there was negligible difference in $IC_{50}$s and maximal signals. The $IC_{50}$s were 1-3 ng/ml indicating that use of the same percent of control urine for the preparation of standard curves may allow us to accurately detect unknown samples using these dilutions.

## Assay validation

The assay validation was performed with the one-step KinExA method. Since the stability of conjugated Cy5 was not affected by incubation with sample during equilibrium, the one-step method was the first choice for this experiment. Recovery tests were conducted with a human urine sample which was spiked with PBA standard solution at 0, 0.5, 2.5, 5, 10, 15, and 20 ng/mL. Linear regression analysis of the results showed a good correlation between spiked and detected levels (Figure7). Average recoveries were ranged between 88 and 160% of the spiked values with $r^2$ of 0.99. These results demonstrated that this assay is able to detect the pyrethroid metabolite at trace levels in urine samples. Biomonitoring of human urine samples conducted by Centers for Desease Control and Prevention during 2001-2002 revealed that measurable amount of PBA (0.3 ng/mL) was found in people of age 6 and older. It should be noted that when the set of measurements was conducted four times successively with no change of reagents (equivalent to 48 samples), we observed significant false positive results from the 3rd measurement indicating that photobleaching of fluorescent dye occurs during prolonged use. One way to avoid this phenomenon is to use freshly prepared fluorescent-labeled antibody solution after every one or two sets of measurement. In addition, if this system is to be used for the analysis of many samples, more intensive efforts should be put into an estimation of urine matrix effect because urine samples of individual persons may have different matrix effects that would result in false positive or negative detection.

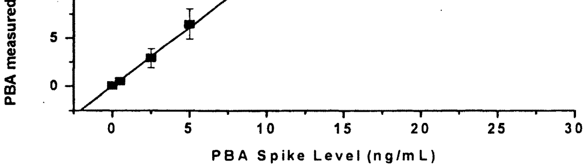

*Figure 7. Relationship between spiked PBA in urine and measured by one-step KinExA.*

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## Conclusion

Development of sensitive one- and two-step noncompetitive assays with the bench top immunoanalyzer, KinExA 3000 system for the detection of a urinary biomarker, PBA in human urine samples is described in this study. Unlike the typical ELISA method, the ability of the KinExA system to separately capture free PBA antibody from the PBA-antibody complex while the sample passed through the bead column greatly improved assay sensitivity. This homologous type of KinExA method eliminates the need of laborious synthesis of competing haptens. It also eliminates the optimization of coating antigen concentration once a sufficient amount of homologous hapten is immobilized on the solid support.

Optimization of coating antigen is frequently carried out by a two-dimensional checkerboard titration method in ELISA. For the proof of the concept, we used a PBA PAb. In our previous study, this antibody showed approximately 100-fold difference in $IC_{50}$s between heterologous and homologous antigens (*27*). The comparison of sensitivities between KinExA methods and ELISAs showed that two homologous KinExA methods had several-fold better $IC_{50}$ values than heterologous ELISAs. The $IC_{50}$ value of the one-step KinExA was a 650-fold improvement over the homologous ELISA. In addition to enhanced sensitivity, another advantage of this system is the high degree of PC-based automation that allows ones to easily set parameters to modulate the injection amount and flow rate of buffer or reagent solution for the best sensitivity eliminating the manual multi-step washings and incubations of ELISA. Once the system is optimized for a target compound, the KinExA consistently performs so that many samples could be consecutively analyzed providing real time results without constructing additional standard curves. One sample could be analyzed within a few minutes. In the case that many samples are to be analyzed with the KinExA, although the one-step assay is fast and more sensitive, the two-step assay is advantageous. A freshly prepared GAR-Cy5 solution can be easily replaced by simply changing out the reservoir with a new one, which prevents the fluorescent dye from photobleaching during the prolonged incubation.

The shape of the KinExAgram is similar to that of a surface plasmon resonance (SPR) assay (*37*). However, the main difference is that the KinExA system requires fluorescent labeling of the reporter molecule whereas the SPR system utilizes a sensor chip-based label-free detection system. The SPR system is among the various types of biosensors that have been reported for the detection of environmental substances (*1, 38*). The main principal of a biosensor is to immobilize recognition elements (e.g., enzyme, antibody, or receptor) on the surface of sensor material connected to the signal transducer. For consecutive analysis, sensor material must be regenerated after one measurement or replaced with new one, making this approach less attractive for high throughput analysis.

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KinExA uses relatively cheap polymers for the immobilization of recognition elements (e.g., antibody or coating antigen). KinExA's fluidic system back-flushes the used material after one measurement is finished and accurately introduces controlled amounts of the new batch of beads. For example, the 200 mg of PMMA beads used in this study allowed 36 consecutive sample analyses. In addition, covalent immobilization of coating antigen is unnecessary for this system. PMMA beads could be coated and blocked within 2 h. In this study, it was important to immobilize on the beads, a sufficient amount of coating antigen with high affinity for antibody to capture all free antibody when the mixture of antibody and analyte pass through the bead bed.

The assay was tolerant to various pH values and ionic strengths. Urine matrix effects were somewhat observed in PBS buffer containing 5 and 10% urine. However, except that the $IC_{50}$ values were slightly increased, good curve shapes and reproducible $IC_{50}$ values of 1-3 ng/ml were obtained. This indicates that when the same amount of control urine is added for the preparation of the standard curve, unknown urine samples could be analyzed. Further optimization may be necessary to meet the relevant concentration range in the prescence of urine. A good correlation was observed between the recoveries by KinExA and spiked level.

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## Chapter 12

# Optical Immunosensor and ELISA for the Analysis of Pyrethroids and DDT in Environmental Samples

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An optical immunosensor (AQUA-OPTOSENSOR) and ELISA (enzyme-linked immunosorbent assay) for the analysis of pyrethroids and DDT in river water and/or sediment, are described. The optical immunosensor consists of a bench-top optical read-out-device and disposable single-use sensor chips. ELISA was carried out in the coating antigen format. As examples, phenothrin (pyrethroid) and *p,p'*-DDT were chosen. Herein we describe the overall strategy, the set-up and principle of the immunosensor platform, and show represent-tative results for immunosensor and ELISA analysis. The immunosensor employs fluorophore (Oyster®-645)-labeled

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monoclonal antibodies (mouse mAb Py-1 and rat mAb DDT 7C12), and makes use of the evanescent field, thus operating without washing steps. ELISA in the coating antigen format uses a second antibody labeled with peroxidase. Both, phenothrin and *p,p*'-DDT can be analyzed with these immunochemical techniques in the low ppb levels. Advantages and drawbacks of both immunochemical platforms are discussed.

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## Introduction

Contamination of different bodies of water with pesticide residues occur during application and runoffs. Some pesticide residues are also persistent and can be found throughout the year. Especially in developing countries, where most communities living in slum areas use untreated water for drinking and for domestic activities, there is an urgent need to provide cost-effective tools for monitoring and control of these contaminations. In addition, new EU directives, such as the Water Framework Directive (WFD; (*1*)), ask for monitoring programs, which will not be affordable, when conventional analytical methods (e.g., LC, GC) will be used. Immunochemical methods, either as immunoassays or as immunosensors, are seen as analytical techniques, which can be established for screening and monitoring scenarios, especially when many samples should be monitored for a limited number of analytes, and when a fast result is needed. In a recent review, the increasing significance of antibody based methods for environmental and food analysis is evident (*2*). In comparison to immunoassays, which still need a lot of pipetting and which are therefore prone to manual mistakes, the advantage of immunosensors is the higher degree of automation. For this purpose, a new optical immunosensor platform (AQUA-OPTOSENSOR) was employed, which uses monoclonal antibodies (mAbs) labeled with a fluorophore (Oyster®-645). Here we describe and demonstrate this new technique together with conventional ELISA (coating antigen format) for the analysis of pyrethroids (*3*) and DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane] (*4*) in different environmental samples. Although DDT has been banned in many countries since 1972 (*5*), it is still present in the environment, especially in tropical countries. Advantages and limitations of this immunosensor in comparison to ELISA will be discussed.

## Materials and Methods

### Chemicals, Reagents, Instruments

Pestanal® standard phenothrin (mixture of isomers, 94.9%) was purchased from Riedel de Haën (Seelze, Germany; now Sigma-Aldrich, Taufkirchen, Germany). DDT isomers and metabolite standards were purchased from the Institute of Organic Industrial Chemistry (Warsaw, Poland). Stock solutions were prepared either in methanol or in ethanol with concentrations of 1 mg/mL and stored at 4 °C. 3-Phenoxybenzoic acid (3-Pba ($C_{13}H_{10}O_3$, $M_r$ 214.2), Fluka), dimethylformamide (DMF, Fluka), *N*-hydroxysuccinimide (NHS, Aldrich), 1,3-dicyclohexylcarbodiimide (DCC, Aldrich), bovine serum albumin (BSA, fraction V powder), ovalbumin (OVA), keyhole limpet hemocyanine (KLH), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich, Taufkirchen, Germany. The derivative of DDT (4-{4-[1-(4-chloro-phenyl)-2,2,2-trichloroethyl]phenyl}butanoic acid ($C_{18}H_{16}C_{14}O_2$; $M_r$ 406.14)), was synthesized by Solvias AG, Basel, Switzerland. All buffer salts, hydrogen peroxide (30% w/w), and solvents were purchased from Merck (Darmstadt, Germany). Pierce Slide-A-Lyzer® dialysis cassettes ($M_r$ cut-off 10,000) and ImmunoPure goat anti-mouse IgG, (Fc), peroxidase (HRP) conjugated, were purchased from Perbio Science Deutschland GmbH, Bonn, Germany. Goat anti-rat-HRP was from Dianova GmbH, Hamburg, Germany.

Ultra pure water was prepared by purifying demineralized water in a Milli-Q (MQ) filtration system (Millipore, Eschborn, Germany) and used for preparation of all buffers. Washing steps were carried out with the automated microtiter plate washer ELX405R (Bio-Tek Instruments, Bad Friedrichshall, Germany). Absorbance of microtiter plates was read with a ThermoMax microtiter plate reader (Molecular Devices, Palo Alto, USA) at 450 nm (reference 650 nm). Data evaluation was carried out with Softmax Pro (Molecular Devices, Palo Alto, USA).

### Preparation of Hapten-Protein Conjugates for Coating Antigens

The 3-Pba hapten and the DDT-derivative (DDT-hapten) were conjugated to different proteins. 3-Pba was conjugated to BSA and OVA as described by Karu et al. (*6*). Briefly, 0.202 mmol NHS and 0.223 mmol DCC were dissolved in 1 mL DMF. This solution was added to 0.200 mmol 3-Pba and stirred for 2½ h at room temperature (RT, 20-23 °C). To remove the precipitated urea, the solution was centrifuged at 1400 rpm for 10 min (Heraeus Sepatech Biofuge 15,

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Heraeus, Hanau, Germany). The active ester was then added slowly to 50 mg BSA and to 50 mg of OVA in 5 mL 0.05 M borate buffer, pH 7.9, respectively. The solutions were stirred overnight at 4 °C. The next day, solutions were transferred into Slide-A-Lyzers and dialyzed for 3 days against 0.02 M PBS (phosphate buffered saline), pH 7.6.

The conjugation of the DDT-hapten to OVA, KLH and BSA was performed accordingly and is described by Weber et al. (*4*). In DDT-hapten-OVA¼, only 0.05 mmol of DDT-hapten were used (instead of 0.2 mmol).

## Preparation of MAb-Oyster®-645 Conjugates

Oyster® dyes (Denovo Biolabels GmbH, Münster, Germany) are available in NHS-ester form and have a molecular weight of about 1000 (NHS-ester). The wavelengths for excitation and emission are as follows: free dye $\lambda_{ex}$ 645 nm, $\lambda_{em}$ 666 nm; protein conjugate $\lambda_{ex}$ 650 nm, $\lambda_{em}$ 669 nm. Prior to conjugation, mAbs were dialyzed against 50 mM sodium borate buffer, pH 7.8. MAb concentrations: Py-1 1.09 mg/mL, DDT 7C12 1.5 mg/mL. Twenty µL of DMF was added to the fluorophore (1 vial of activated dye contains an amount to label 1.5 mg of protein). One aliquot (2 µL) of the dissolved dye is added directly to the different mAb solutions of Py-1 and DDT 7C12 (containing 300 µg of protein each). The vials were shaken continuously at ambient temperature. The degree of labeling of mAbs is controlled by the incubation time. After 30 min incubation (for Py-1) and after 10 min incubation (for DDT 7C12), the reaction was blocked by adding 2 µL of 10% (w/v) glycine per 100 µL of antibody solution. MAb-Oyster®-645 conjugates were dialyzed in Slide-A-Lyser cassettes against 10 mM phosphate buffer, pH 7.6. They were stored in the dark at 4 °C until used.

## AQUA-OPTOSENSOR – General Principle and Set-up

The AQUA-OPTOSENSOR system is based on a formerly developed immunosensor (*7, 8*) and consists of a bench-top optical read-out-device (Siemens AG, Munich, Germany; Figure 1, left), and a disposable single-use low-cost sensor chip (pes Diagnosesysteme GmbH, Leipzig, Germany), including a fluidic system and an internal piston pump (Figure 1, right). The sensor chip consists of an optical prism (polymethyl methacrylate, PMMA, 11 mm x 23 mm), which is mounted to a carrier via an adhesive tape. The tape forms in addition the height of the flow channel. The internal pump controls the transport of the fluid. The valve and the pump are interfaced with a cock drive and a plunger, the latter being a part of the bench-top device.

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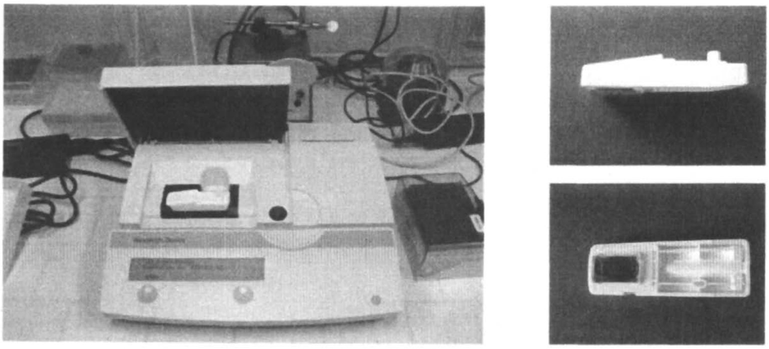

*Figure 1. AQUA-OPTOSENSOR set-up. Bench-top instrument and one way sensor chip. Left side: instrument with open lid; right side top: one one-way chip from the side; right side bottom: one-way chip bottom with PMMA prism.*

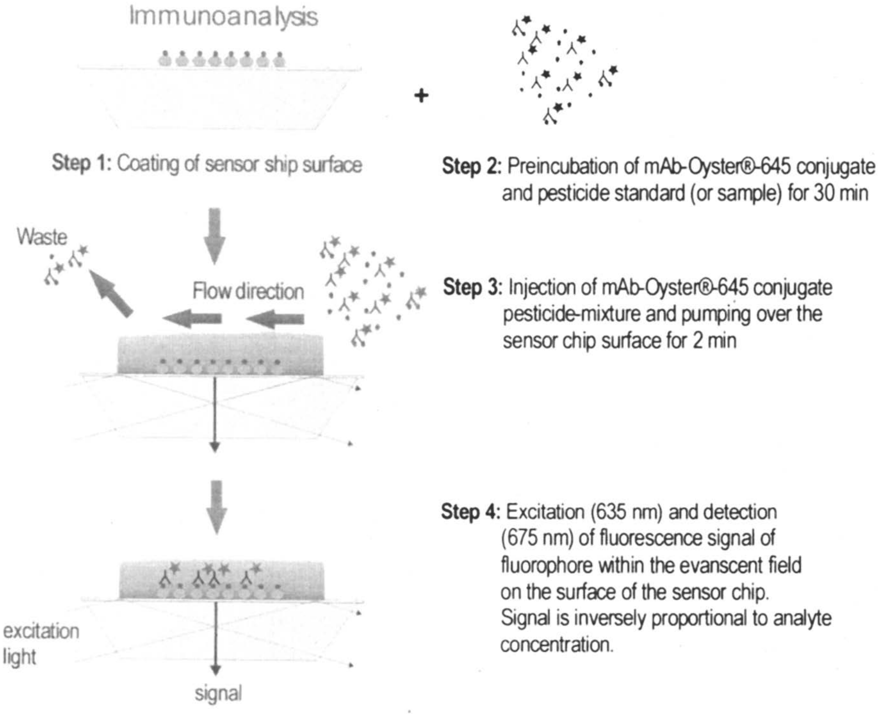
Immunoanalysis
+
Step 1: Coating of sensor ship surface
Step 2: Preincubation of mAb-Oyster®-645 conjugate and pesticide standard (or sample) for 30 min
Waste
Flow direction
Step 3: Injection of mAb-Oyster®-645 conjugate pesticide-mixture and pumping over the sensor chip surface for 2 min
Step 4: Excitation (635 nm) and detection (675 nm) of fluorescence signal of fluorophore within the evanscent field on the surface of the sensor chip. Signal is inversely proportional to analyte concentration.
excitation light
signal

*Figure 2. AQUA-OPTOSENSOR - Details of the principle for immunoanalysis of pesticides.*

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The pesticide-protein conjugate is adsorbed onto the surface of the prism (Figure 2). The fluorophore labeled anti-pesticide mAb is typically pre-incubated for 30 min with the pesticide standard or sample. Then the mixture is pipetted into the charging hole of the sample container and pumped over the surface of the chip (2 min). The fluorophore is exited via a laser beam ($\lambda_{ex}$ 635 nm) and the fluorescence emission of the fluorophore labeled antibody is measured in an photomultiplier (($\lambda_{em}$ 675 nm). The signal (slope [mV/s]) is registered via the AQUA-OPTOSENSOR software (Siemens AG, Munich, Germany), and is inversely proportional to the analyte concentration. Data transfer and evaluation is done either in MS Excel or in SigmaPlot. Standard curves were fitted according to the four-parameter equation: y = ((A-D)/ (1 +(x/C)^B)) + D.

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## AQUA-OPTOSENSOR for Pyrethroids (Phenothrin) or for DDT

The 3-Pba-BSA conjugate (4.35 mg/mL; 0.1 µL) or DDT-hapten-KLH conjugate (3.1 mg/mL, 0.1 µL) were adsorbed on the surface of the prism. The prism was fixed to the sensor chip via an adhesive tape and was ready to be used.

A mixture of mAb Py-1-Oyster®-645 conjugate (0.6 µg/mL in 2% (w/v) BSA/40 mM PBS) and phenothrin standard (0.1 to 100 µg/L in 40 mM PBS) or a mixture of mAb DDT 7C12-Oyster®-645 conjugate (2 or 5 µg/mL in 2% (w/v) BSA/40 mM PBS) and *p,p'*-DDT (0.05 to 100 µg/L in 5% (v/v) isopropanol in 40 mM PBS) was pre-incubated for 30 min (with shaking, at RT) prior to the injection into the sensor chip. Successive steps were the same as described under the general principle.

## ELISA for Pyrethroids (Phenothrin)

Microtiter plates were coated with 250 µL/well of 3-Pba-BSA (2.5 µg/mL in 0.05 M sodium carbonate buffer, pH 9.6) and incubated overnight in the refrigerator (4 °C). The plates were washed (4 mM PBST, pH 7.6), and 100 µL/well of phenothrin standards (0.01 to 10,000 µg/L in 40 mM PBS, pH 7.6) were added, followed by 100 µL/well of mAb Py-1 (109 ng/mL in 40 mM PBS, pH 7.6). This mixture was incubated for 2 h at RT, with shaking of plates on a plate shaker. Plates were washed again, and 200 µL/well of goat anti-mouse mAb conjugated with HRP (1:40,000 in 40 mM PBST, pH 7.6 were added and incubated for 1 h at RT. After another washing step, 200 µL/well of substrate solution (0.4 mM TMB, 1.3 mM $H_2O_2$ in 100 mM sodium acetate, pH 5.5) were added, incubated for 10-30 min, stopped with 50 µL/well of 2 M $H_2SO_4$, and read at 450 nm (reference 650 nm) in the plate reader. For better comparison with other standard curves, the absorbance values were normalised to %control according to the formula: Control (%) = ($A/Ao$) x 100, where $A$ is the value of the absorbance of each standard and $Ao$ is the value of absorbance for the zero standard (40 mM PBS).

## ELISA for DDT

Microtiter plates were coated with 200 µL/well overnight at 4 °C at final concentrations of DDT-hapten-OVA¼ (1 µg/mL) in 0.05 M sodium carbonate buffer, pH 9.6. The next day, plates were washed three times with 4 mM PBST (PBS, pH 7.6, with 0.05% Tween 20). A blocking step with milk powder (1% (w/v) in 40 mM PBS pH 7.6, 250 µL/well) was used with an incubation of 1 h at RT. After a washing step with 4 mM PBST, 100 µL/well of *p,p'*-DDT (0.01 to 10,000 µg/L were prepared in 40 mM PBS, pH 7.6, from a stock solution in methanol) and 100 µL/well of mAb DDT 7C12 (125 ng/mL in 40 mM PBS) were added successively and incubated for 2 h at RT. After another washing step with 4 mM PBST, 200 µL/well of goat anti-rat-HRP (1:20,000 in 40 mM PBST, pH 7.6) were added and incubated for 1 h at RT. After a final washing step, 200 µL/well of substrate solution (TMB/$H_2O_2$ in 100 mM sodium acetate buffer, pH 5.5) were added. Color development of the enzymatic reaction was stopped after 10 or 15 min with 50 µL/well of 2 M $H_2SO_4$. The absorbance was read at 450 nm (reference 650 nm). Standard curves were normalised to %control as described in the previous section. Zero standard corresponds to 40 mM PBS.

## Water Sample Sources for Pyrethroids (Phenothrin) or *p,p'*-DDT Analysis

For the screening of pyrethroids and *p,p'*-DDT in river water, water samples were obtained and collected during a field trip (Sept. 2005; EU-project AQUA-SCREEN) from different sampling points (starting from the river head: Ondire) along the Nairobi River, Kenya. For this first demonstration, water samples were analyzed in the laboratory with the AQUA-OPTOSENSOR. PBS (40 mM) was analyzed as a control. In addition, a selection of these water samples has been spiked with 1 µg/L of phenothrin to show the specific reaction.

In addition, for the demonstration of the screening for *p,p'*-DDT in water samples, SWIFT-WFD1 and SWIFT-WFD2 were used. These water samples had been collected in April 2005 from the water monitoring station in Eijsden, The Netherlands (Maas River, EU-project SWIFT-WFD) during field experiments by project partners (Dr. Nathalie Guigues, BRGM, Orleans, France; Mr Ian Allan, University of Portsmouth, UK). These water samples were sent to our laboratory and then analyzed with the sensor. Another water sample was

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taken from the sampling point Loretto of the Nairobi River. In addition, water samples were spiked with different concentrations of *p,p'*-DDT. 40 mM PBS was analyzed as a reference and compared with the different water samples.

These demonstrations were used to show how the immunosensor could be used as a screening method for different sources of water samples.

All water samples from the sampling points along the Nairobi River were also analyzed with ELISA for phenothrin and *p,p'*-DDT in the laboratory.

## Sediment Sample Preparation, ELISA, and GC-MS-Analysis

Sediments samples were also collected from the same sampling points of the Nairobi River, and samples from three sampling points were analyzed by ELISA and GC-MS in the laboratory.

Wet sediment sample (2-3 g) were weighed in a 100 mL centrifuge tube with teflon-sealed screw cap. Copper (1 g) and 10 mL acetone were added and extracted for 2 h in an ultrasonic bath (Sonorex RK 510S, Bandelin, Berlin, Germany). The sample was shaken every 15 min during extraction. The sample was centrifuged at 2000 rpm for 10 min. Ten mL of the clear extract were separated, evaporated to dryness by a gentle stream of nitrogen and reconstituted in 1 mL n-hexane.

Silica gel (1 g, deactivated with 1.5% $H_2O$) and 1 g of $Na_2SO_4$ were weighed in a 6 mL cartridge. The cartridge was washed with 6 mL 30% dichloromethane (DCM) in n-hexane. The sample extract was drawn on top of the column. The analytes were eluted with 9 mL 30% DCM in n-hexane and adjusted with n-hexane to 10 mL. An aliquot of 5 mL was concentrated (under nitrogen stream) to nearly dryness, then 0.5 mL isopropanol was added. This extract was diluted 1:25 with 40 mM PBS for ELISA, and the corresponding standard curves were carried out in 4% isopropanol (40 mM PBS).

Another 5 mL aliquot was taken for GC-MS-analysis, 10 µL internal standard and 150 µL n-nonane were added. The eluate was concentrated under a gentle stream of nitrogen to nearly 100 µL and transferred to a glass micro vial.

GC-MS-analysis was performed using an 8000 GC-MS-system from Fisons (Rodano, Italy), consisting of a Fisons GC8000, a CTC auto sampler AS200 and a quadrupole mass-spectrometer MD800. The capillary column used was a pesticide II (Restek) with a length of 30 m, an internal diameter of 0.32 mm and a film thickness of 0.25 µm. Helium was used as carrier gas with a constant column head pressure of 50 kPa and an initial flow rate of 1.2 mL/min. A volume of 1 µL was injected in splitless mode. The following temperatures were used: injector temperature was set at 220 °C, and the detector interface temperature was maintained at 300 °C. The temperature gradient was: 80 °C for 1 min, ramped with 12 °C/min to 140 °C and with 10 °C/min to 300 °C. The

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mass selective detector was operated in EI-SIM mode using the *m/z* ions listed in Table I. Five-point internal calibration was used in the concentration range from 10 to 500 pg/µL for native analytes and a constant internal standard concentration of 100 pg/µL.

**Table I. Selected masses in EI-SIM analysis of DDT related compounds**

| *Substance* | *m/z* *native* | *m/z* *labelled* |
|---|---|---|
| *o,p'*-DDT | 235/237 | |
| *o,p'*-DDE | 246/248 | |
| *o,p'*-DDD | 235/237 | |
| *p,p'*-DDT | 235/237 | 247/249 |
| *p,p'*-DDE | 246/248 | 258/260 |
| *p,p'*-DDD | 235/237 | |

## Results and Discussion

During many monitoring studies, it will be necessary to get a swift overview of the contamination. Samples will be collected, transported to the laboratory, and then analyzed immediately. Alternatively, samples might be even analyzed on-site, thus saving the transport. In both situations immunochemical methods, such as the optical immunosensor and the ELISA described herein, will be very useful tools. As examples, we will show some screening situations for environmental samples: surface water and/or sediment.

### AQUA-OPTOSENSOR and ELISA for Pyrethroids (Phenothrin) and DDT

For the analysis of pyrethroids, phenothrin was always used as standard. A typical standard curve with AQUA-OPTOSENSOR for phenothrin was obtained with a test midpoint ($IC_{50}$) at about 2 µg/L and a working range from 0.2-20 µg/L (Figure 3, solid line). For the analysis of *p,p'*-DDT with mAb DDT 7C12, standard curves with a working range from 0.5-30 µg/L and an $IC_{50}$ at about 5 µg/L were obtained (Figure 3, dashed line). This shows that both insecticides can be analyzed at the low ppb levels.

The analysis of phenothrin by ELISA in the coating antigen format revealed a standard curve with an $IC_{50}$ of about 2 µg/L and a working range from 0.2-20 µg/L (Figure 4, solid line). This result is comparable to the immunosensor.

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The standard curve for p,p'-DDT in ELISA was steeper than in the optical sensor, therefore the working range was quite narrow (1-10 µg/L; Figure 4, dashed line). The $IC_{50}$ though was lower than in the optical sensor (about 2 µg/L versus 5 µg/L).

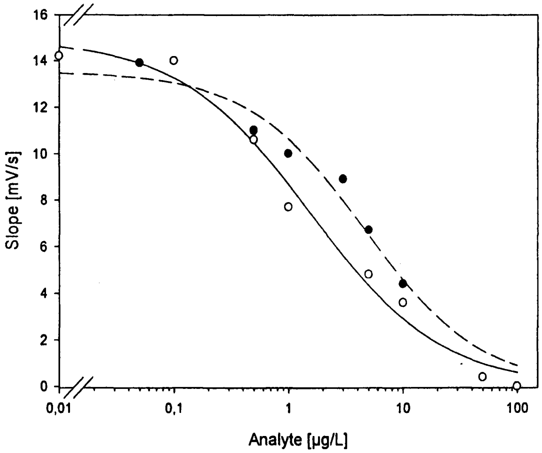

*Figure 3. AQUA-OPTOSENSOR - Standard curves for phenothrin (mAb Py-1-Oyster®-645) and p,p'-DDT (mAb DDT 7C12-Oyster®-645 2 µg/mL in 2% (w/v) BSA/40 mM PBS). Curve fitting (SigmaPlot): ○ solid line (phenothrin): A= 14.9; B= 0.76; C= 1.5 µg/L; D= 0.0; $R^2$= 0.986; ● dashed line (p,p'-DDT): A= 13.5; B= 0.86; C= 4.6 µg/L; D= 0.0; $R^2$= 0.975.*

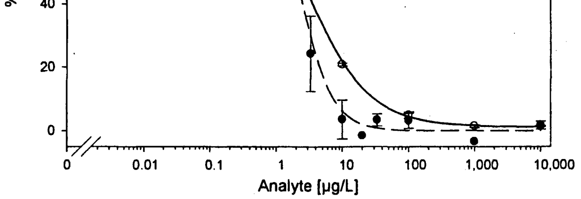

*Figure 4. ELISA (coating antigen format) - Standard curves for phenothrin (mAb Py-1) and p,p'-DDT (mAb DDT 7C12). Curve fitting (SigmaPlot): ○ solid line (phenothrin): A = 97.4; B = 0.893; C = 2.32 µg/L; D = 1.22; $R^2$ = 0.999 (n=3, intra-day); ● dashed line (p,p'-DDT): A = 95.5; B = 1.7; C = 2.1 µg/L; D = 0.0; $R^2$ = 0.988 (n=2-4, inter-day). 0 µg/L = 40 mM PBS.*

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### Screening of Water Samples for Phenothrin and p,p'-DDT with AQUA-OPTOSENSOR and ELISA

Water samples from different sampling points along the Nairobi River were collected. Using the AQUA-OPTOSENSOR for the analysis of phenothrin, screening results obtained from the water were compared with the measurement of 40 mM PBS. As seen in Figure 5, water samples were comparable to the result obtained with 40 mM PBS, thus revealing that phenothrin was not present. Only the water sample from Ondire showed a slight inhibition, which might indicate the presence of phenothrin or another cross reacting pyrethroid. This was also confirmed by ELISA (data not shown).

To demonstrate the specificity for phenothrin, the water samples were spiked with 1 μg/L of phenothrin. When phenothrin is present, a clear inhibition is visible with all water samples (Figure 5).

Water samples from the sampling points of the Nairobi River were also screened for the presence of *p,p'*-DDT (Figure 6). With the optical immunosensor the samples were analyzed in comparison to 40 mM PBS as a control. No significant decrease of signal was visible up to sampling point 7 (Chiromo), which indicated that either *p,p'*-DDT is not present or present below the detection limit (0.5 μg/L). An inhibition was visible from sampling points 8-11. The highest inhibition was observed for the sample Outerring Road (about 50%), which corresponds to 4 μg/L of *p,p'*-DDT (Figures 3 and 7)).

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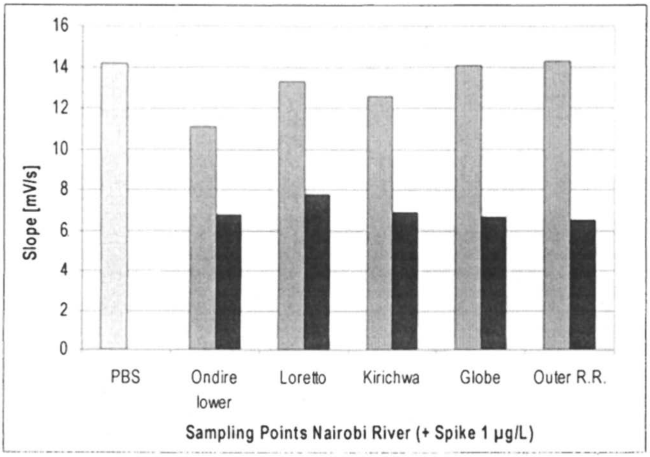

*Figure 5. AQUA-OPTOSENSOR - Analysis of Nairobi River water samples from different locations (▨) for phenothrin (mAb Py-1) plus spikes of 1 μg/L (■), in comparison to 40 mM PBS (□).*

Comparing these results with those of ELISA (Figure 7), the concentrations for *p,p'*-DDT in the water of these sampling points (8 to 11) are in the same range (2-3.3 µg/L).

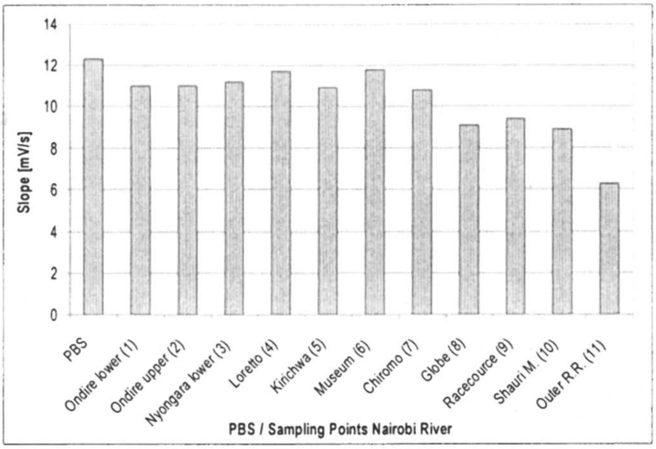

*Figure 6. AQUA-OPTOSENSOR - Analysis of Nairobi River water samples for p,p'-DDT (mAb DDT 7C12)*

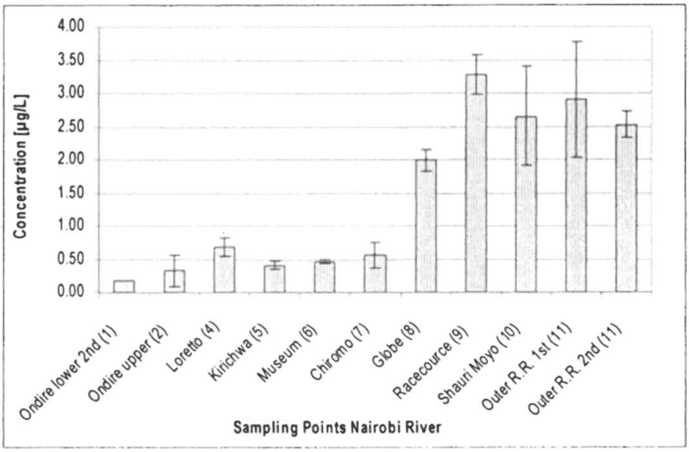

*Figure 7. ELISA - Analysis of Nairobi River water samples for p,p'-DDT (mAb DDT 7C12). Sampling point Outerring Road (11) 1$^{st}$ and 2$^{nd}$ refers to two different sampling dates, both in Sept. 2005.*

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Although *p,p'*-DDT is also banned in Kenya and other tropical countries, it is still applied sometimes because DDT is the most cost effective insecticide to control mosquitos, reducing the rate of malaria (*9, 10*). Immunochemical techniques could be then very useful tools to control contamination after spraying and minimize the risk to human health.

As another demonstration of the screening potential of the sensor, we spiked several concentrations of DDT into different water matrices (two samples from the Maas River, The Netherlands; one sample from sampling point Loretto, Nairobi River, Kenya). A clear inhibition with increasing *p,p'*-DDT concentrations was observed (Figure 8). PBS (40 mM) was compared directly with the spiked water samples.

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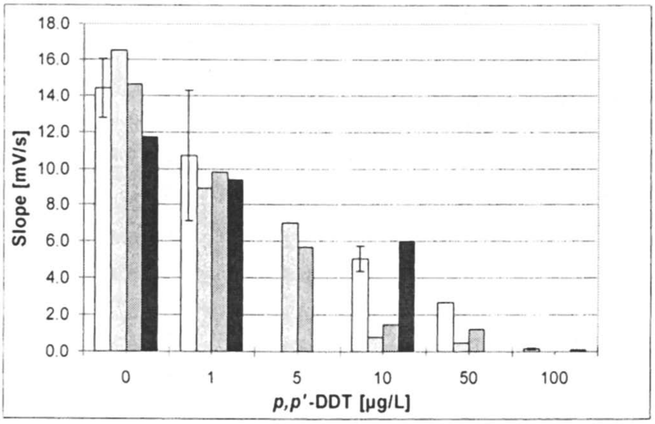

*Figure 8. AQUA-OPTOSENSOR - Screening of water samples from different sources for p,p'-DDT. 40 mM PBS was used as a reference value (n=3-4, inter-day). 0 µg/L = 40 mM PBS or different unspiked water samples. Columns: PBS (□); SWIFT-WFD1(▨), SWIFT-WFD2 (▩;both Maas River, The Netherlands); Nairobi River, Kenya, location Loretto (■).*

## Analysis of Sediment Samples for DDT by ELISA in Comparison to GC-MS

Three sediment samples from the sampling points along the Nairobi River were analysed for DDT compounds. Extracts were split and analysed by ELISA and GC-MS. GC-MS results shown in Table II indicate a trend in concentration: Ondire < Museum < Outerring Road. This corresponds well to the results obtained with the water samples from the same sampling points.

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**Table II. GC-MS Analysis for DDT and Related Compounds**

| | *GC Result [ng/g]* | | | | | |
|---|---|---|---|---|---|---|
| Compound/ Sampling Point | *p,p'*- DDT | *o,p'*- DDT | *p,p'*- DDD | *o,p'*- DDD | *p,p'*- DDE | *o,p'*- DDE |
| Ondire | 0.81 | 0.52 | 0.72 | 0.43 | 1.74 | 0.00 |
| Museum | 2.59 | 0.89 | 1.28 | 0.55 | 2.08 | 0.23 |
| Outerring R. | 5.55 | 2.26 | 4.41 | 1.86 | 4.22 | 0.64 |

MAb DDT 7C12 has a higher recognition for *p,p'*-isomers (*p,p'*-DDT 100%; *p,p'*-DDD 98%, *p,p'*-DDE 51%, *o,p'*-DDT 56%, *o,p'*-DDD 15%, *o,p'*-DDE 6%). Despite this fact, about 70% of the total amounts of DDT isomers and metabolites detemined by GC are expected to be recognized by ELISA (Table III, columns 2 and 3). Yet, the amounts which were determined by ELISA were 2.6 to 7.6 times higher than the expected values in respect to GC-MS and the CRs of these mAbs (Table III, column 5).

**Table III. Comparison of Results for DDT Obtained by GC-MS and ELISA (Coating Antigen Format)**

| *Sampling Point* | *Determined by GC/MS [µg/L]* | *Sum of DDT expected ELISA [µg/L]* | *Ratio expected* $\frac{ELISA}{GC}$ | *Determined by ELISA [µg/L]* | *Ratio* $\frac{Determined}{Expected}$ *ELISA* | *Determined* $\frac{ELISA}{GC}$ *[%]* |
|---|---|---|---|---|---|---|
| Ondire | 4.22 | 2.76 | 0.65 | 21 | 7.6 | 497 |
| Museum | 7.62 | 5.50 | 0.72 | 17 | 3.1 | 223 |
| Outer R.R. | 18.94 | 13.62 | 0.72 | 35 | 2.6 | 185 |

NOTE: The calculation model used (additive model) was kindly provided by Dr. Robert Harrison, CAPE Technologies, L.L.C., South Portland, Maine, USA

NOTE: Compounds determined by GC/MS, see Table II

Concentrations determined by ELISA were always higher than by GC-MS, probably due to matrix interferences of the sediment samples. The concentrations of ELISA using the enzyme-tracer format were 53-118% respective to GC-MS (data not shown), whereas those of coating antigen format were 185-497% respective to GC-MS (Table III, column 6). The reason for this is probably that the enzyme-tracer format can detect DDT in lower concentrations, thus allowing to dilute the sediment extract 1:50 instead of 1:25.

### AQUA-OPTOSENSOR versus ELISA - Advantages and Drawbacks

The AQUA-OPTOSENSOR and the coating antigen ELISA differ quite significantly in their set-ups. Although a coating antigen is used in both systems, the mAbs are either used labeled (sensor) or unlabeled (ELISA). In addition, the optical sensor makes use of the combination of light excitation with a laser beam for a specific fluorophore and the usage of the evanescent field. This fact enables a very selective measurement mode, which does not need washing steps for separation of bound and unbound material. Only the bound fraction of the (labeled) antibodies is detected, all other unbound mAbs and also potential fractions of the environmental matrix, which might fluoresce, are not seen by the system. The coating antigen on the surface of the PMMA chip is used about 1000 times more concentrated than usually applied on the surface of the wells of microtiter plates (mg/mL in comparison to µg/mL range). The labeled mAbs and the sample are pre-incubated, so the coating antigen is then only used to catch those labeled mAbs, which did not bind during the incubation to pesticide molecules in the standard or sample (Figure 2). Therefore the incubation time on the surface of the PMMA chip can be kept very short (2 min); this is also advantageous to avoid unspecific binding.

In contract to ELISA no parallel determination of standards and samples can be carried out on the same chip of the sensor system. This means that the presision of the sensor measurement is very much dependent upon the resproducibility of the PMMA surface and the coating procedure.

Although the results of the optical immunosensor and the ELISA are comparable, a drawback of the sensor system is that measurements can only be carried out successively. Therefore, the analysis of many samples is much faster done by ELISA, whereas the AQUA-OPTOSENSOR is faster for a smaller number of samples.

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## Conclusions

In environmental analysis many monitoring situation exist, which ask for cost-effective strategies. Within the EU Water Framework Directive, monitoring programmes should be in place by the end of 2006. For the implemention of this Directive these monitoring programmes will be very important and will not be affordable, if conventional analytical methods will be applied. Therefore there is an urgent need to have cost-effective and reliable screening methods.

Another urgent need is identified in developing countries, where a part of the population lives in slum areas and uses untreated surface water for drinking and domestic activities. Again, there is an urgent need to provide cost-effective tools for screening and control of pesticide residues, especially during and after applications.

The use of untreated water calls in addition for fast and easy methods to monitor pathogens. The herein described optical sensor can also be employed with nucleotides as selective recognition elements. This is another set of applications, where the sensor platform would be very useful.

Using these techniques, it will be possible to establish a strategy for pollution monitoring in water matrices. This strategy should combine the low-cost and fast immunosensor and/or immunoassay screening together with the time- and cost-intensive laboratory analysis.

## Prospects

In this work, the AQUA-OPTOSENSOR and the conventional ELISA were used for the analysis of only one analyte per chip and microtiter plate, respectively. Generally though, it is possible to adapt these platforms for multi-analyte analysis. Currently, up to three analytes can be determined, both in the sensor and on one plate. Three spots on the single-use chip (representing three different coating antigens) were pipetted on the PMMA surface. On the plate, wells were coated with different coating antigens. This was demonstrated for the parallel analysis of isoproturon, diuron and phenothrin (*11, 12*). The strategy has to take into consideration that the pairs of coating antigens – mAbs used do not cross-react with each other.

The sensor also has application for nucleotide detection making it ideal for new applications such as detection of agents of bioterrorism.

## Acknowledgments

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## Chapter 13

# Immunological Determination of the Pharmaceutical Diclofenac in Environmental and Biological Samples

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A highly sensitive and specific competitive ELISA on 96-microwell plates was developed for the analysis of the non-steroidal anti-inflammatory drug diclofenac. Within the water cycle in Europe, this is one of the most frequently detected pharmaceutically active compounds. The LOD at a signal-to-noise ratio (S/N) of 3, and the $IC_{50}$, were found to be 6 ng/L and 60 ng/L respectively in tap water. In a comparative study using ELISA and GC-MS, diclofenac levels in wastewater from 21 sewage treatment plants were determined and a good correlation between these methods was found (ELISA vs. GC-MS: r = 0.70, slope = 0.90, intercept = 0.37, n = 24). An average degradation rate of ≈25% can be calculated. Lab-scale-experiments on the elimination of diclofenac in continuous pilot sewage plants revealed a removal rate of only 5% over a period of 13 weeks. In a further study, the ELISA was applied to a number of extracts of various animal tissues from a range of species, and again a very good relationship between ELISA and LC-ESI/MS data sets was obtained ($r = 0.90$, $p<0.0001$; $n = 117$). The ELISA has proven to be a simple, rapid, reliable and affordable alternative to otherwise costly and advanced techniques for the detection of diclofenac in matrix diverse water samples and tissue extracts after only relatively simple sample preparation.

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*Abbreviations*: ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection; CV, coefficient of variation; NSAID, nonsteroidal anti-inflammatory drug; GC-MS, gas chromatography mass spectrometry; LC-ESI/MS, liquid chromatography electrospray ionisation mass spectrometry; WWTP, wastewater treatment plant; ds, dry sludge; TG, thyroglobulin; OVA, chicken egg albumin; MeOH, methanol; EtOH, ethanol; DMSO, dimethyl sulfoxide; MeCN, acetonitril; TMB, tetramethylbenzidine, BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; GaRIgG-POD, goat anti-rabbit IgG-horseradish peroxidase conjugate; MTP, microtiter plate; PBS, phosphate buffered solution; SPE, solid phase extraction; HDPE, high density polyethylene; COD, chemical oxygen demand; $BOD_5$, 5-day biochemical oxygen demand; DOC, dissolved organic carbon, TNb, total nitrogen bound; CR, cross-reactivity; $IC_{50}$, inflection point of the sigmoidal calibration curve.

## Introduction

During the last two decades, a considerable number of reports have been published on the widespread occurrence of residues of pharmaceuticals in the environment, i.e. in aquatic and soil compartments (*1-7*). Up to now, more than 100 compounds have been identified. These may originate from both human and veterinary usage and can be released directly after passing through wastewater treatment plants (WWTPs), after sludge disposal on farm land, and by other sources such as landfill leachates and nonpoint discharges from agricultural runoff (*8, 9*). Current knowledge about the long-term effects of low-level exposure to such compounds is limited, and chronic effects may not become apparent for many years. In a wider sense, potentially serious environmental effects are often neglected, e.g. impacts on non-target species are practically unknown (*10, 11*).

Diclofenac belongs to the most frequently detected pharmaceutically active compounds in the water-cycle in Europe. Average concentrations of diclofenac in the low ppb range were detected in influents and effluents of municipal WWTPs and surface waters in Austria, Brazil, Finland, Germany, Greece, Spain, Switzerland, United Kingdom, and the United States (*5, 6, 12-14*). Approximately 80 tons of this nonsteroidal anti-inflammatory drug (commonly referred to as an 'NSAID') are sold annually in Germany alone (*2*).

Recently, several very significant environmental effects of this pharmaceutical have been documented. In the aquatic compartment, the feminization of freshwater fish has been noted (*15*), and renal changes can occur in trout exposed to environmental concentrations of diclofenac (*16-18*). Besides this, a lethal impact has now been reported on wild bird populations (*26, 27*). Diclofenac was introduced to the veterinary market on the Indian subcontinent (India, Nepal, and Pakistan) during the early 1990s, and it is currently widely used in the treatment of inflammation, pain and fever in livestock. Since the introduction of diclofenac to this market, once common *Gyps* vulture populations (*Gyps bengalensis, Gyps indicus and Gyps tenuirostris*) have apparently declined by >97% (*19-22*). These three *Gyps* species are now listed as Critically Endangered (*23-25*), yet in the mid-1980s, *Gyps bengalensis* was considered to be one of the most common large birds of prey in the world. It appears that vultures have been scavenging prevalent diclofenac-contaminated ungulate carcasses and that diclofenac is highly toxic towards all *Gyps* species so far tested (*26, 28*). This is now considered to be the major cause of perhaps the most rapid avian species population decline in recorded history. Consequently, a ban on the veterinary use of diclofenac in livestock is being actively sought, and a captive vulture breeding programme has been established in India.

As part of this vulture breeding program, a continuous and reliable 'diclofenac-free' source of ungulate carcasses is required for feeding purposes. Unfortunately, sourcing meat known to be drug free has proved difficult and expensive. Therefore, a rapid, inexpensive, easy to use analytical technique is required by those involved in this conservation effort in India and elsewhere. In utilizing such a technique, on site, in breeding centres, captive vultures can be

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fed meat known to be diclofenac-free, and the risk of accidental poisoning can be minimized.

In general, pharmaceuticals are designed to produce specific biological effects on organisms. For the most part, they tend to be very stable and highly water soluble. As a result of this, many drugs are rather persistent in the environment, i.e. they show incomplete biodegradation and also low sorption onto sediments and soil components. This in turn favours their mobility through the WWTP and allows them to stay in solution once they have entered receiving surface waters. These micropollutants represent only a minor part of the wastewater organic load, and the majority of such compounds and their metabolites cannot even be detected at present since the analytical methods with low enough limits of detection have not yet been developed. Several technical options for removing pharmaceuticals from wastewater are currently under discussion, and these are mainly focussed on the optimization and upgrading of existing treatment technologies, e.g. improving biological treatment and ozonation of the effluent (*29*). In the long term, new approaches incorporating better waste stream design and source separation (i.e. separation of different household wastewaters) will undoubtedly gain increased interest (*30*).

In the case of diclofenac, reported elimination rates in WWTPs and biofilm reactors run from almost zero (*31-33*) to about 75% (*2, 34*), and even up to quantitative (100%) elimination (*48*). After ozonation of a WWTP effluent, diclofenac was no longer detected (*29*). Advanced oxidation of the drug was also observed when concentrated solutions were treated with UV-light in a photoreactor or with UV/$H_2O_2$ and ozone (*35, 36*). Strenn *et al.* studied in detail the elimination of diclofenac in lab-scale plants working with different sludge retention times, and in 12 Austrian WWTPs (*37*). Removal rates of 0-74% were observed and no dependency on the sludge retention time was indicated.

As with other pharmaceuticals, both gas and liquid chromatography combined with MS(-MS) detection are commonly applied to the analysis of diclofenac (*38*). Immunochemical techniques can provide an interesting alternative approach for analyzing pharmaceuticals, by taking advantage of highly selective compound binding by antibodies. Whereas a great number of immunoassays have now been developed and used for the analysis of pesticides which are found at similar levels in aquatic systems, surprisingly, very few tests have been applied to pharmaceutical compounds thus far. Only a few groups have shown the feasibility of adapting clinical assays to the analysis of water samples for the presence of pharmaceuticals, and these have tended to focus on hormones and anticancer drugs (*39-45*).

In this article, we report on the development of a very sensitive and selective ELISA for diclofenac, and on its use for the analysis of wastewater samples to estimate the concentration of this pharmaceutical in different WWTPs in Germany and Austria. Further, laboratory experiments were performed to

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evaluate the elimination rate of diclofenac under very controlled conditions. Finally, the practicality of using this ELISA to measure diclofenac in extracts from different animal tissues/species is also investigated.

## Materials and Methods

All reagents were of analytical grade unless otherwise specified. The sodium salt of diclofenac (2-(2,6-dichlorophenyl]amino)benzeneacetic acid), chicken egg albumin (OVA), porcine thyroglobulin (TG), gelatin, milk powder, casein, tributylamine, isobutyl chloroformate, 1,4-dioxane, methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide (DMSO) and acetonitrile (MeCN) were purchased from Sigma-Aldrich (Munich, Germany). Tetramethylbenzidine (TMB), 30% hydrogen peroxide ($H_2O_2$) and Tween 20 were obtained from Merck (Darmstadt, Germany). Goat anti-rabbit IgG-horseradish peroxidase conjugate (GaRIgG-POD) ad keyhole limpet hemocyanin (KLH) were purchased from Pierce (Rockford, USA). Bovine serum albumin (BSA) was from Serva Feinbiochemica (Heidelberg, Germany). Freund's complete and incomplete adjuvants were obtained from Difco Labs (Detroit, USA). β–Glucuronidase/arylsulfatase solution was obtained from Roche Applied Science (Mannheim, Germany).

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### Antiserum Production

The antiserum was prepared as reported recently (*46*). Briefly, after converting diclofenac sodium salt into the free acid it was coupled to BSA and KLH by the mixed anhydride method. Primary immunization of two rabbits was performed intradermally at 10 multiple sites by injecting the diclofenac-KLH immunogen emulsified with Freund's complete adjuvant. Booster injections were administered over a period of 28 weeks after priming using Freund's incomplete adjuvant. After the first booster, an immunogen switch was performed and further immunizations were done with the diclofenac-BSA conjugate. Rabbits were bled through the ear vein. Serum samples obtained from the final bleeding, 3 weeks after the last booster injection, were split into 1-mL aliquots and stored frozen in liquid nitrogen. The serum from the rabbit which showed the highest titer and sensitivity was used in this study.

### ELISA Procedure

An indirect competitive ELISA format on microtiter plates (MTP) was developed for the analysis of diclofenac (*46*). MTP were coated with the coating antigen (diclofenac-TG conjugate, 20 ng/mL; 200 µL/well) in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The plates were covered with adhesive plate sealing film to prevent evaporation. After overnight incubation at 4 °C, the plates were washed four times with PBS-Tween (0.01 M PBS, pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20) using an automatic plate washer. Binding sites not occupied by the coating antigen were blocked with blocking buffer (PBS containing 1% casein; 300 µL/well) for 1 h at room temperature. Plates were then washed as before. For construction of the calibration curve a diclofenac stock solution (0.5 g/L) was prepared with methanol and then further diluted with pure water to obtain standard solutions which covered the concentration range between 0.01 and 10 µg/L. Samples or standard solutions (100 µL/well) and diluted rabbit antiserum (1:20 000 in PBS; 100 µL/well) were added and incubated at room temperature for 1 h. After washing four times with PBS-Tween, GaRIgG-POD was added (1:8000 in PBS; 200 µL/well), incubated at room temperature for 1 h and plate washed as before. The substrate solution (TMB/$H_2O_2$) was prepared by mixing TMB stock solution in DMSO (1%, w/v, 100 µL), $H_2O_2$ (5%, 10 µL), substrate buffer (0.1 M sodium acetate buffer, adjusted to pH 5.8 by adding 1 M citric acid solution, 500 µL) and pure water (10 mL). Substrate solution (200 µL/well) was added and the plates were agitated for about 15 min for color development. Finally, the enzyme reaction was stopped with sulphuric acid (5%; 100 µL/well) and the absorbance was read at 450 nm with a plate reader. All determinations were made at least in triplicate. The sigmoidal standard curves were set up using Rodbard's four-parameter function and were plotted in the form of $B/B_0 \times 100$ (%) against log C (where B and $B_0$ were the values of absorbance measured at the standard concentrations and at zero concentration, respectively).

The relative sensitivity of the immunoassay towards 14 pharmaceuticals, 6 diclofenac metabolites, and 6 pesticides of similar chemical structure was determined by assaying a dilution series of each compound in water containing 10% of methanol (for fenoprofen, mefenamic acid and tolfenamic acid, due to their lower solubility in 10% MeOH, they were assayed in 10% DMSO). All chemicals were tested in the concentration range of 0.001 to 1000 µg/L. The $IC_{50}$ values (concentration of inhibitor that produces a 50% decrease of the maximum normalized response) were compared and expressed as a percent $IC_{50}$ based on 100% response to diclofenac.

## GC-MS Procedure

Analysis of wastewater samples was done at two different laboratories using slightly modified procedures as previously reported (*46*). Consistently, the sample volume was 1 L, and the pH of the filtered water sample was adjusted to

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about pH 2 with 1 N HCl to enhance trapping of diclofenac on the SPE sorbent (Strata C18-E or Bondesil C18). The derivatization was carried out using a 2% solution of pentafluorobenzylbromide in cyclohexane or toluene, and addition of 2 or 3 µL of triethylamine to the dry sample, followed by heating at 90 or 100 °C for 1 to 2 h. As internal standards, either desmetryn or 2,3-dichlorophenoxyacetic acid and 2-(2,4-dichlorophenoxy)propionic acid were added. After cooling, this solution was analyzed by GC-MS (gas chromatograph equipped with a split-splitless autoinjector and coupled to a quadrupole mass spectrometer (Agilent, Palo Alto, CA)). Separations were carried out by means of a HP 1701 or HP-5MS column (each 30 m x 0.25 mm i.d., film thickness 0.25 µm) with Helium as the mobile phase. The injection volume was 2 µL. The mass spectrometers were operated in SIM-mode and for quantitative analysis, m/z ratios 313 and 475 or 214, 216, and 242 were used.

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## Water Samples

Wastewater samples were made available from 21 WWTPs in south Bavaria, and effluent samples from fifteen WWTPs in Austria, taken between September and November 2002. The WWTPs are connected to sewage systems which service regions containing between 1,000 and 2 million residents, and each utilizes at least three commonly used treatment steps (i.e, preliminary and final clarification and an aerator tank). All the samples were analyzed on the day of sampling or stored overnight in the fridge and analyzed on the following day, (in order to minimize microbial degradation). Analysis was performed after filtration (using Whatman GF/C glass microfiber filters) to remove particles >1.2 µm. The filtrates were then diluted with pure water (minimal dilution of 1:10) and applied to the ELISA procedure.

## Lab-Scale-Experiments

The biodegradation of diclofenac was investigated in a continuous pilot sewage plant used as a model system for municipal sewage treatment. Synthetic wastewater was used, which was composed of 144 mg/L peptone, 99 mg/L of meat extract, 27 mg/L of urea, 6.3 mg/L NaCl, 25.2 mg/L $K_2HPO_4$ and 4 µg/L diclofenac. In Figure 1, one of the six pilot sewage plants used is shown. These bioreactors operate as a conventional activated sludge system with nitrogen-elimination. Each consists of a synthetic wastewater influent, a non-aerated denitrification reservoir, an activated sludge tank, a final sedimentation tank, and an effluent. Using an upstream non-aerated reservoir and optimal settings for denitrification (i.e. low exposure of the activated sludge to degradable compounds and internal recirculation of nitrificated wastewater), constant nitrogen elimination in the sewage plants was ensured. The oxygen content in the activated sludge tank was held at 2-3 mg/L. To attain final sedimentation, settled activated sludge was discontinuously recirculated in the denitrification reservoir. Excess sludge was then removed every second day.

Four diclofenac dosed and two control (no dose) test reactors were utilised. After a week of an initial break-in phase (utilising synthetic wastewater only) the test reactor influent was spiked with 4 µg/L diclofenac and then the effluent was monitored for the following thirteen weeks. Influent samples were taken once, and effluent samples twice a week and analyzed by ELISA after a twentyfold dilution with distilled water. For comparison, two samples from days 75 and 90 were also analysed by LC-ESI/MS after enrichment of diclofenac using SPE. In order to assess the functional efficiency of the sewage plants, in addition to diclofenac, the parameters COD (Chemical Oxygen Demand), $BOD_5$ (5-day Biochemical Oxygen Demand), DOC (Dissolved Organic Carbon) and TNb (Total Nitrogen bound) were also measured.

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### Tissue Samples

Diclofenac levels were determined by both LC-ESI/MS and ELISA in MeCN extracts from a range of sources:

- In liver and kidney tissue extracts of ungulates (cow (*Bos indicus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*)) collected in the field and at carcass dumps in India (n = 27; plus n = 31 'blanks'). Collected as part of an on-going survey into the prevalence of diclofenac in vulture food sources.
- In liver, brain, muscle, kidney, blood and bile extracts of vultures (*Gyps africanus*) from South Africa (n = 24; including 6 'blanks'); and kidney and liver extracts from vultures (*Gyps fulvus*) from Spain (n = 12). These birds were experimentally treated with diclofenac under license in order to investigate diclofenac toxicity to different *Gyps* species.
- In muscle, liver, blood, intestine and kidney extracts of cow and goat from experimentally treated animals in India (n = 21); utilized to investigate diclofenac pharmacokinetics.
- In liver and kidney extracts of vultures (*Gyps* sp.) found dead in the field in Nepal and India (n = 5).

In total, 120 extracts were analyzed in this study, including 37 blank samples (genuine tissue extracts found to be diclofenac free by LC-ESI/MS). A range of extract concentrations between 9 and 1561 µg/L (by LC-ESI/MS) were utilized.

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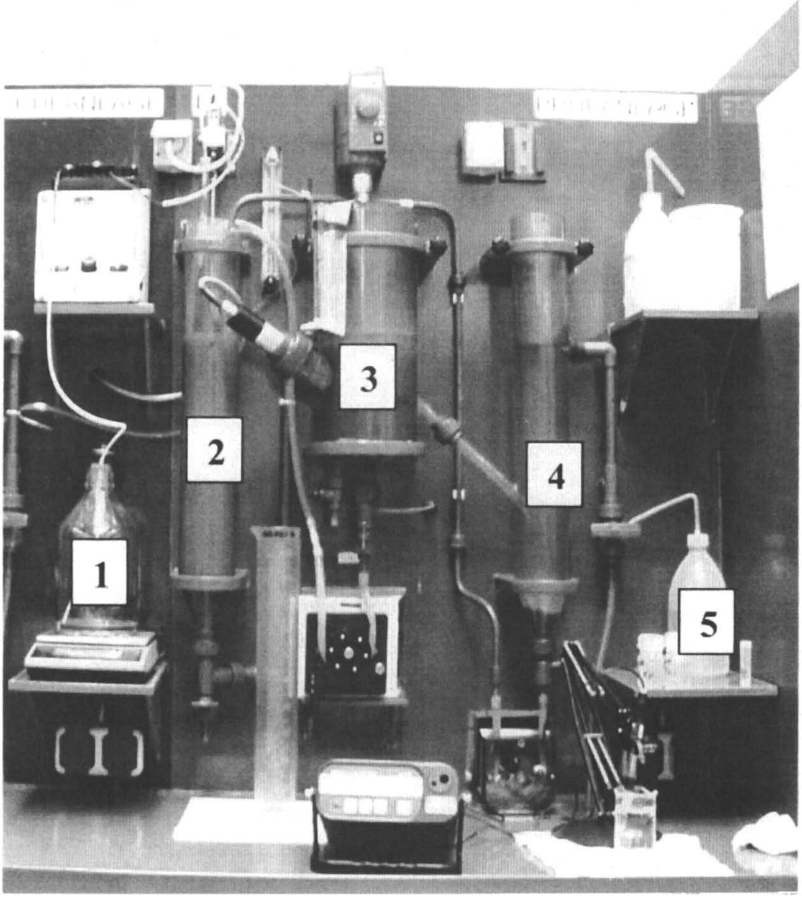

*Figure 1. Pilot sewage plant setup: 1, synthetic wastewater influent; 2, non-aerated denitrification reservoir; 3, activated sludge tank; 4, final sedimentation tank; 5, effluent*

*Tissue sample collection and preparation.*

Samples were from a variety of sources and as such differed in the nature of their collection. Field collected samples of vultures and ungulates were taken opportunistically (especially in the case of vultures), and during planned sampling surveys at carcass dumps across India (in the case of cow, buffalo and goat). In both cases, these tissues may have undergone some degree of decomposition before collection. Upon collection, sample details (site, tissue type, sex, age, etc) were routinely recorded and approximately 20 g of tissue was placed in a labelled HDPE (high density polyethylene) container and frozen (at -20 °C) until extraction. Experimentally treated tissues were collected soon after death/slaughter and were, as such, known to have undergone little decomposition before freezing. A number of experimentally treated tissues were 'aged' at ambient temperature following death in order to determine whether any changes in diclofenac in certain tissues thence occurred (replicating field conditions where ungulates die and are then scavenged at some point following variable degrees of decomposition).

Extraction of diclofenac from tissues was achieved using 0.500 ± 0.001 g of tissue, extracted using 2 ml of HPLC grade MeCN. The tissue was weighed into a new glass test tube, MeCN added, and the mixture homogenized for 30 seconds using an Ultra Turrax IKA T8 Homogenizer (except in the case of the Nepalese vulture samples, which were homogenized manually by pestle and mortar). The mixtures were then centrifuged at 2000 rpm for five minutes and the supernatant filtered using disposable PTFE/PE syringe filter units of 0.45 μm. The filtered extract was then stored in crimp top LC-MS vials at -20 °C until analysis. Aliquots were diluted at least 1:500 with pure water and then applied to the ELISA procedure. Depending on the diclofenac concentration, additional dilutions of, 1:1000, and 1:5000 had to be prepared in order to bring the concentrations into the working calibration range of the ELISA.

## LC-ESI/MS procedure

LC-ESI/MS determination of diclofenac was achieved using an Agilent 1100 series LC-ESI/MS (1946D). The instrument was calibrated using 7 standards ranging from 5 to 1000 μg/L in diclofenac concentration, generated using diclofenac sodium salt. The calibration was linear across this range with an $r^2$ value of at least 0.99. Both a blank and a mid-range standard were analysed every 10 samples in order to monitor for instrumental drift and/or diclofenac carry-over. Diclofenac was monitored by the MS at mass/charge ratios of 294 and 296 (the deprotonated and protonated ions) in the negative ion mode. The instrument was calibrated against both masses and each sample ran through the

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instrument twice; this allowed four values for diclofenac to be gained (at 294 and 296 in two runs), the mean of which was taken to be the concentration in the extract. The limit of detection for this technique (back calculated to wet tissue concentration) was found to be 5 μg/kg.

Chromatographic separation was achieved on the LC using a Waters Xterra MS C18 column (3.9 mm x 150 mm, 5 μm). Samples and standards (20 μL) were subjected to a binary gradient elution profile using 0.1% acetic acid in water (solution A) and 100% MeCN (solution B). The starting conditions were 75% A/25% B for 0.1 min, followed by a 15 min linear gradient from 75% A/25% B to 5% A/95% B, then by a 5 min column wash step of 5% A/95% B, and a 10 min re-equilibration step with 75% A/25% B. The flow rate was set at 0.7 mL/min, samples were continuously held cool at 4 °C using an autosampler with thermostat, and the column temperature was maintained at 40 °C during analysis. The mass spectrometer acquired data in the negative ion mode, with the capillary voltage set at 3500 V and the fragmentor voltage at 80 V.

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## Results and Discussion

### ELISA Optimization

For the development of a highly sensitive ELISA, the assay conditions, such as the concentration of the immunoreagents (coating antigen, antiserum and GaRIgG-POD), the blocking reagent, and the effect of temperature and organic solvents should be carefully optimized. In this study, the criteria used to evaluate the optimization were: maximum absorbance ($B_0$), dynamic range, $IC_{50}$ and detection limit (LOD). It was found herein, that the best combination of immunoreagents was 20 ng/mL of diclofenac-TG conjugate for coating, and dilutions of 1:20000 for antiserum and 1:8000 for the GaRIgG-POD conjugate. Casein at a concentration of 1% was optimal for blocking, i.e, the background signal was well below 10%. Therefore, plates were routinely coated at 4 °C overnight followed by a blocking step at room temperature for 1 h.

The diclofenac calibration curve was constructed over a concentration range of 0.01 to 10 μg/L. A typical curve is shown in Figure 2. The relative standard deviation of the measured absorbance for three replicates at each standard concentration was consistently lower than 5%. The LOD at a signal-to-noise ratio (S/N) of 3, and the $IC_{50}$, were found to be 6 ng/L and 60 ng/L, respectively. The analytical working range (i.e, the linear part of the curve between 20% and 80% of inhibition) was between 20 and 400 ng/L. For ten standard curves consecutively performed during a two week period, the $IC_{50}$ varied between 40 and 80 ng/L (inter-assay CV of 18.7%).

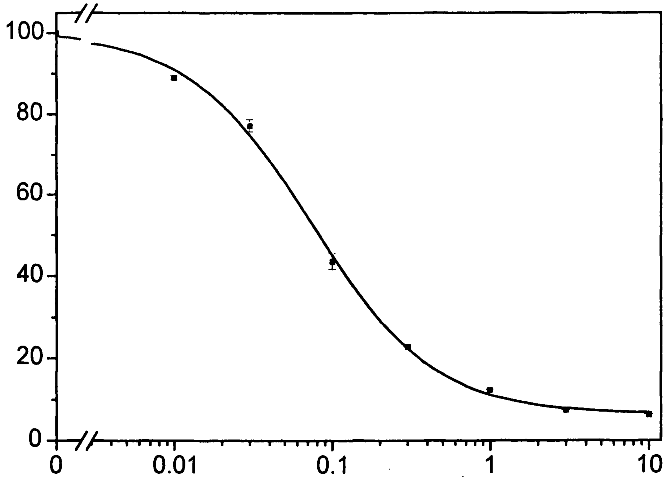

*Figure 2. Diclofenac standard curve obtained with spiked pure water samples. Error bars represent ±1 standard deviation about the mean (Reproduced from reference 46. Copyright 2003 American Chemical Society)*

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Antiserum specificity was tested by cross-reactivity (CR) measurements. The highest CR detected amongst 26 compounds tested was found to be 100% for the metabolite 5-hydroxydiclofenac, which is structurally very similar to the analyte (Figure 3). CR values for the other compounds tested were well below 4% and are individually negligible. Any substituents at the dichlorophenyl ring lead to a significant loss in antibody binding. This finding clearly demonstrates that, under optimized conditions, the developed ELISA is highly specific for diclofenac and its 5-hydroxy derivative.

The tolerance of the ELISA to the four organic solvents, MeOH, EtOH, DMSO and MeCN, was also tested. For this, a series of diclofenac standard solutions were prepared with these organic solvents at concentrations of 2 to 30%. With increasing concentration of the organic solvent the $IC_{50}$ value of the standard curve tended to increase, the rate/extent of increase depended on the solvent used (see Table I). Results indicated a gradual shift in the standard curve at higher diclofenac concentrations and, therefore, a loss in sensitivity was observed. A significant increase in the $IC_{50}$ was observed for MeOH, EtOH or DMSO at concentrations above 20%, whereas even 10% MeCN lead to a significant loss in sensitivity.

## Analysis of Wastewater Samples

All samples were filtered and diluted with pure water before being analyzed by ELISA. It was found that a 10 times dilution with pure water was completely

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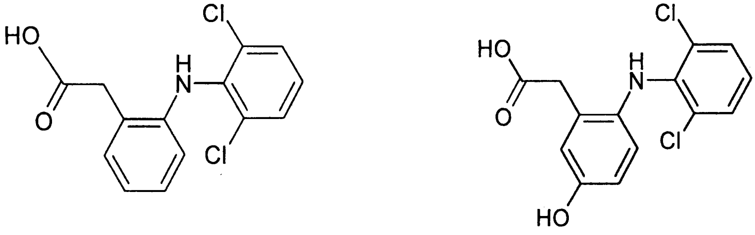
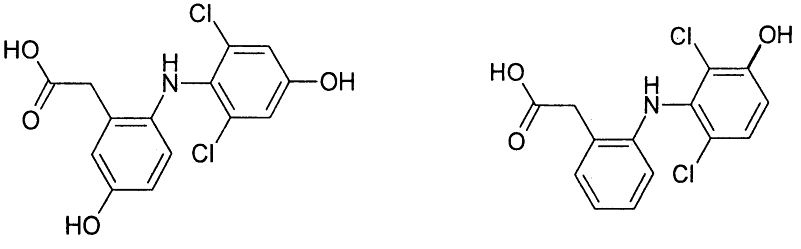
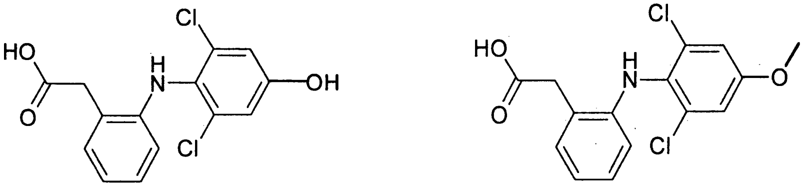

diclofenac
(100%)

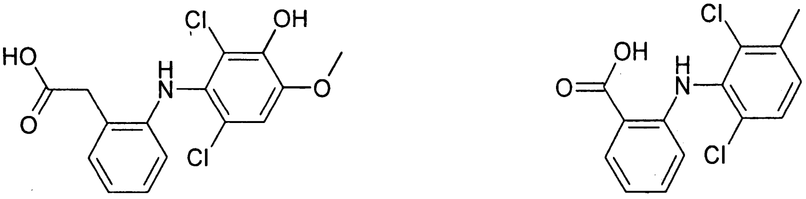
diclofenac (primary)
metabolite (3-hydroxy derivative)
(100%)


diclofenac metabolite
(5,4'-dihydroxy derivative)
(1.5%)

diclofenac metabolite
(3'-hydroxy derivative)
(0.6%)


diclofenac (primary) metabolite
(4'-hydroxy derivative)
(0.4%)

diclofenac metabolite
(4'-hydroxymethyl derivative)
(0.2%)


diclofenac metabolite
(3'-hydroxy-4'hydroxymethyl derivative)
(<0.1%)

meclofenamic acid
(3.5%)

*Figure 3. Chemical structures of target analyte, diclofenac metabolites and meclofenamic acid. Number in brackets corresponds to the percent $IC_{50}$ value based on 100% response to diclofenac (Adapted from reference 46. Copyright 2003 American Chemical Society)*

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

**Table I. $IC_{50}$ Values (μg/L) Along the Diclofenac Standard Curve Using Different Concentrations of Various Organic Solvents**

| *Organic Solvent* | *$IC_{50}$ Values (μg/L)* | | | | | |
|---|---|---|---|---|---|---|
| | *Concentration of Organic Solvent (%)* | | | | | |
| | *0* | *2* | *5* | *10* | *20* | *30* |
| MeOH | 0.05 | 0.06 | 0.06 | 0.08 | 0.10 | 0.14 |
| EtOH | 0.05 | 0.07 | 0.07 | 0.08 | 0.10 | 0.19 |
| DMSO | 0.06 | 0.07 | 0.08 | 0.10 | 0.13 | 0.34 |
| MeCN | 0.05 | 0.05 | 0.08 | 0.18 | 0.43 | 0.98 |

SOURCE: Adapted from reference 46. Copyright 2003 American Chemical Society

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sufficient to eliminate matrix effects. The analysis of surface water samples from rivers and lakes has previously shown an enhanced ELISA response that can be caused by dissolved organic matter (data not shown) (46). As a consequence, samples had to be diluted at least 1:5 before analysis, which is intrinsically tied to a loss in sensitivity. However, in summary, a diclofenac concentration as low as 60 ng/L (LOD) could be reliably detected in wastewater by the ELISA. In an initial study which included 4 WWTPs, higher than average diclofenac concentrations in influent wastewater samples (2.62 ± 0.50 μg/L) compared to effluents (2.00 ± 0.51 μg/L) were observed. From these data an average degradation rate of only 25% could be calculated.

To confirm the widespread presence of the drug in wastewater systems, 24 samples from 21 WWTPs were analyzed both by ELISA and GC-MS. The results are summarized in Figure 4. Regression analysis gave a good linear relationship between the two methods (ELISA vs. GC-MS: r = 0.70, slope = 0.90, intercept = 0.37). However, the average concentration as determined by GC-MS (0.93 ± 0.51 μg/L) was around 25% lower than the corresponding concentration by ELISA (1.22 ± 0.66 μg/L). Several explanations for this finding would be possible. Firstly, higher ELISA values will be found if, beside the parent drug, metabolites and/or drug conjugates or unknown chemicals are recognized by the antibody. In this study, some metabolites were made available as a gift from NOVARTIS Co. Besides 5-hydroxydiclofenac, which showed identical binding to the antibody as the parent molecule, the other metabolites exhibited only a very low CR, below 2% (Figure 3). No data were found in the literature on the presence of and stability of diclofenac glucuronides and sulfates in WWTPs. Glucuronides are known to be rather unstable, depending on the type of conjugate, i.e., esters are less stable than ethers.

For example, Reddy *et al.* reported recently on the presence of steroid sulfates in effluent (47). In this study, enzymatic cleavage of samples using β-glucuronidase/arylsulfatase yielded almost identical results using immunochemical and gas chromatographic methods, i.e. 1.34 ± 0.34 μg/L vs. 1.29 ± 0.16 μg/L (n = 7).

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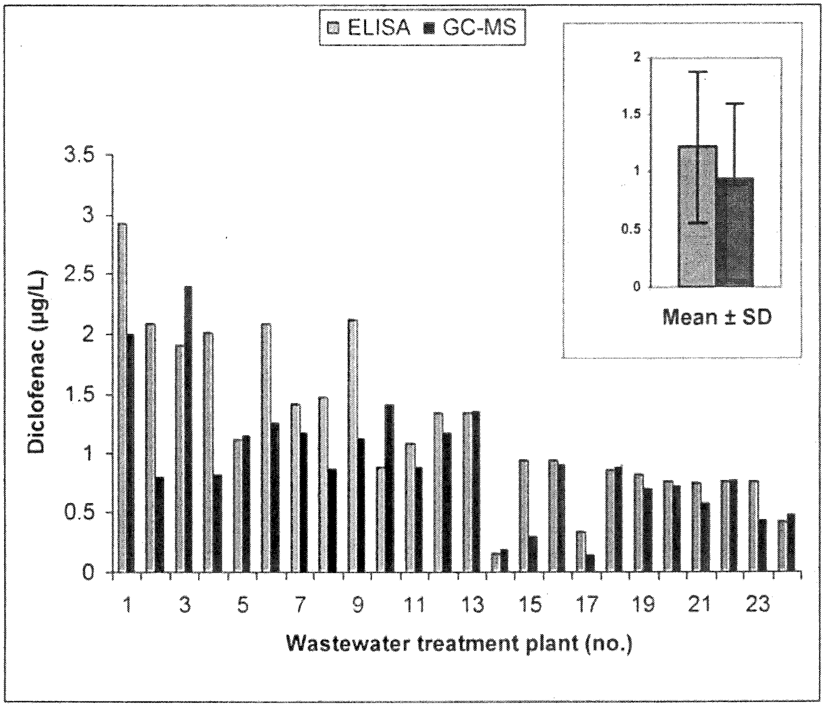

*Figure 4. Method comparison of diclofenac determination in wastewater samples using ELISA and GC-MS. The diagram in the insert shows the mean ±1 standard deviation (n = 24) of 21 WWTPs (Adapted from reference 46. 2003 Copyright American Chemical Society.)*

This might be an indirect indication of the presence of diclofenac metabolite(s) and their binding by the raised antibodies. Investigations are ongoing to determine different diclofenac metabolites in WWTPs using GC-MS.

As a conclusion, the developed ELISA was found to be a simple, inexpensive, and accurate method for the determination of diclofenac in wastewater after rather simple sample preparation. In addition, it was proven that in all investigated WWTPs (n = 21) the pharmaceutical was present in the effluents at an average concentration of approximately 1 µg/L.

## Lab-Scale-Experiments

The elimination (biodegradation and sorption) of diclofenac in four pilot sewage plants was evaluated by comparing the obtained concentration of spiked influent and effluent samples. Figure 5 shows the diclofenac elimination over time. The error bars represent the standard deviation of the four bioreactors. With the exception of the first sample, the concentration of diclofenac was not reduced significantly during sewage treatment in the four plants. The average removal amounted to only 5%.

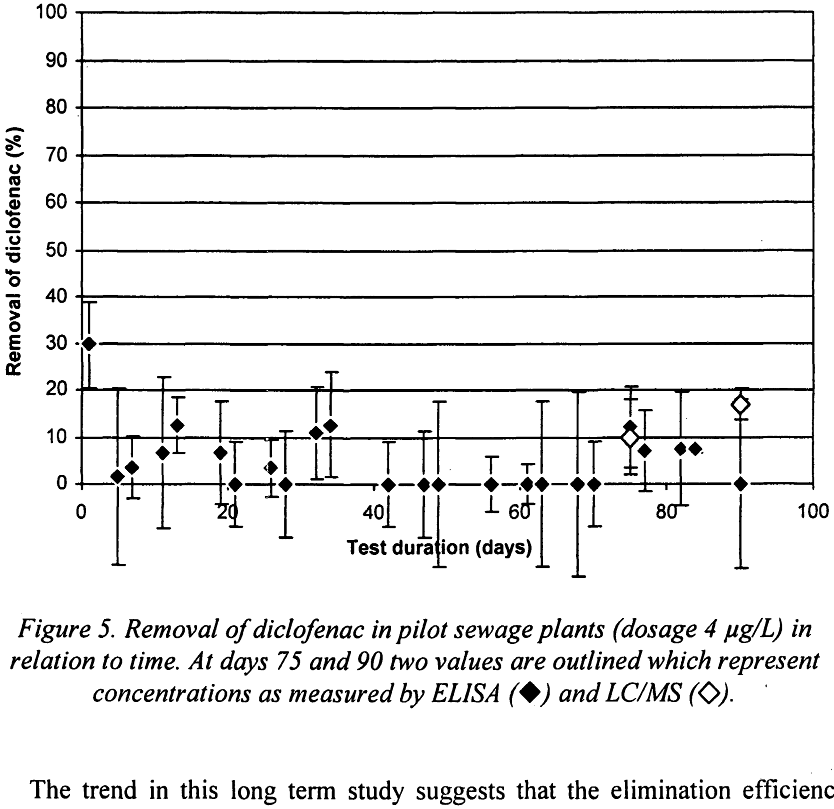

*Figure 5. Removal of diclofenac in pilot sewage plants (dosage 4 µg/L) in relation to time. At days 75 and 90 two values are outlined which represent concentrations as measured by ELISA (◆) and LC/MS (◇).*

The trend in this long term study suggests that the elimination efficiency also remains rather constant. Therefore, no adaptation of the micro biota appears to occur (over a thirteen week period). In addition to biological degradation, sorption is a major elimination pathway during wastewater treatment. To assess the sorption of diclofenac the excess sludge generated was also analyzed. The content of diclofenac varied between 29-267 µg/kg ds with a mean value of 137 ± 67 µg/kg ds (data not shown). No temporal dependency was observed. Hence, it can be concluded that a fast saturation of the sludge takes place and remains at a low level over time. A total of only 0.1% of the diclofenac mass flux becomes sorbed onto the sludge. In summary, diclofenac is not significantly eliminated by activated sludge, neither by biodegradation or sorption.

In order to assess degradation efficiency the elimination of carbon and nitrogen compounds in the test and control sewage plants were determined. All

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pilot sewage plants exhibited good purification efficiency. During sewage treatment carbonaceous compounds were almost quantitatively eliminated (96-98%). The elimination of nitrogen compounds amounted to 77%.

Furthermore, with respect to the efficiency of the test and control plants, no inhibiting effect of diclofenac on the microorganism populations was observed. Both exhibited a decreased COD from 225 mg/L to 10 mg/L, whereas the $BOD_5$ decreased from 116 mg/L to approximately 2 mg/L. The conclusion can be drawn that the used sludge exhibited optimal functionality. Therefore, the absence of diclofenac elimination can not be attributed to inoperable sludge.

In this detailed study it became obvious, that diclofenac is not eliminated to a higher extent by the activated sludge process. No significant biodegradation or sorption could be obtained, even though the sludge exhibited optimal functionality. Since reported elimination rates of diclofenac vary between 0 and 100%, a differentiated examination of the literature data is taken. Including the results of this study it becomes noticeable that in the majority of lab-scale experiments no notable removal of diclofenac was achieved (*32, 37*). In contrast, in some investigations, higher removal rates were calculated by comparing real influent and effluent samples of WWTPs working with conventional activated sludge techniques (*2, 13, 34, 48, 49*). It seems that additional processes in some real plants may be important for the removal of diclofenac. For example, in surface waters, the direct photodegradation of diclofenac has already been described as an important elimination process, generating half-lives of minutes to a few hours in natural waters (*50-55*). If the hydraulic retention time in the WWTP is sufficiently high, photolysis can be a relevant degradation process. Detailed studies regarding photodegradation during sewage treatment are rare, however, a significant photodegradation of antimicrobial agents in wastewater has already been reported (*56*). The photodegradation of diclofenac in wastewater has not been demonstrated thus far and more detailed investigations in this respect are now needed.

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## Biomonitoring

Figure 6 shows the relationship between the LC-ESI/MS and the ELISA data for all 117 samples analyzed during this work (excluding 3 bile samples). As is clear, the overall relationship between the two sets of data are very good ($r = 0.90$, $p < 0.0001$) and as the regression indicates, the relationship between the two techniques approaches 1:1. Given the wide concentration range determined (up to almost 1600 µg/L), the number of different tissue types and species, and the differences in degree of decomposition of the tissue samples analyzed, this represents a very robust set of data. It indicates that this test would be highly applicable to the purpose for which it could be utilized, i.e, testing tissue extracts to ensure they are 'diclofenac-free'.

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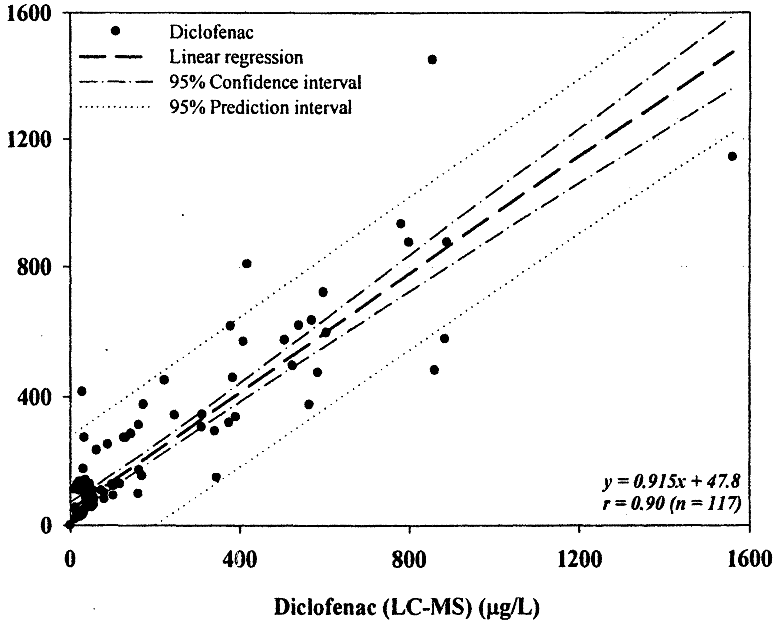

*Figure 6. Comparison between ELISA and LC-ESI/MS analysis of diclofenac (showing the linear regression and the 95% confidence and prediction intervals).*

Indeed, Table II shows that for the 'Indian field collected ungulate' set of data (where n = 58), which may be the most appropriate portion of the data to consider given the proposed end use of the ELISA, the r = 0.92, p <0.0001 and the regression/mean ELISA:LC-ESI/MS ratios all indicate a tight relationship between these two very different analytical techniques.

The ELISA tests were achieved 'blind' of the LC-ESI/MS data, in different laboratories, and at no point did the ELISA return a negative result when the LC-ESI/MS returned a positive, crucial, given the intended use.

However, as a prerequisite, tissue sample extracts had to be diluted at least 1:500 to eliminate matrix interferences, i.e., generating a limit of quantification of 10 µg/L (20 µg/kg of tissue), somewhat higher than that achievable when analysing tap water samples, but comparable to achievable LC-ESI/MS limits.

Table II breaks the overall dataset down by tissue extract type. A number of points should be noted. Where bile was concerned, no reportable data could be provided by the ELISA due to matrix interferences, and further development work would be needed if analysis of this sample type were required. Likewise, the relationship between the ELISA and the LC-ESI/MS data was found not to be significant for the 12 Spanish experimental vultures tested. For the Indian

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Table II. Relationship Between LC-ESI/MS and ELISA by Sample Type Tested

| *Type* | *n* | $r^2$ | *p* | *Regression* | *Mean ELISA:LC-ESI/MS Ratio (SE)* |
|---|---|---|---|---|---|
| Spanish experimental vultures | 12 | 0.14 | 0.2358 | -2.7x + 242 | 3.64 ± 1.01 |
| Indian field collected vultures | 2 | Insufficient data | ~ | ~ | 10.06 ± 2.25 |
| South African experimental vultures - bile | 3 | no relationship obtainable | ~ | ~ | ~ |
| Nepalese field collected vultures | 3 | 0.79 | 0.3014 | 0.83x + 25.7 | 0.97 ± 0.20 |
| Experimental cow/goat tissue - aged | 9 | 0.72 | 0.0036 | 1.32x + 57.5 | 2.14 ± 0.37 |
| Experimental cow/goat blood - fresh | 7 | 0.90 | 0.0012 | 0.54x + 65.7 | 0.86 ± 0.19 |
| Experimental cow/goat tissue - fresh | 5 | 0.97 | 0.0020 | 1.36x + 0.5 | 1.34 ± 0.08 |
| Indian field collected ungulates | 58 | 0.85 | <0.0001 | 1.04x + 30.8 | 1.17 ± 0.13 |
| South African experimental vultures - blood | 9 | 0.97 | <0.0001 | 1.02x - 16.6 | 0.97 ± 0.04 |
| South African experimental vultures - tissues | 12 | 0.98 | <0.0001 | 1.94x - 0.1 | 1.51 ± 0.17 |
| *Overall relationship* | *117* | *0.81* | *<0.0001* | *0.92x + 47.8* | *1.42 ± 0.19* |

field collected vultures, n was only 2, however the ratio between the ELISA:LC-ESI/MS was very high (ELISA gave much higher diclofenac values). This was not the case for the vulture samples collected in Nepal, where the regression was not found to be significant. In this case however, n was only 3 and the raw data did agree reasonably well (LC-ESI/MS gave 32, 564 and 506 µg/L; ELISA gave 38, 376 and 574 µg/L, respectively).

Where discrepancies have been noted to arise between the two techniques, a number of key factors may be relevant. Firstly, matrix interference. Although both techniques are somewhat susceptible to this, ELISA is recognized as being more susceptible in this regard especially when a matrix is complex, i.e, high in a wide range of organic compounds. The exact nature of matrix interference in ELISA analysis is still, however, relatively poorly understood. Interferences may be compounded by tissue age (note the elevated mean ratio between the two techniques for 'aged' experimental tissues and field collected Indian vultures) as the matrix may be more complex for an extract of partially decomposed tissue.

Secondly, there may be an effect caused by the differences in the diclofenac specificity of the two techniques. The LC-ESI/MS is highly compound specific, whereas the ELISA will respond not only to diclofenac, but also to other closely related compounds (see cross-reactivity in the section on *ELISA Optimization*). This particular ELISA is, for example, 100% cross-reactive with the 5-hydroxydiclofenac metabolite, one of the two primary diclofenac metabolites currently identified which are formed by the cytochrome P450 system. It may be, therefore, that in fresh tissue (Spanish and South African experimental vultures), the ELISA is responding to diclofenac plus a significant level of the 5-hydroxy metabolite (or another as yet unidentified highly cross-reactive metabolite). This in itself is worthy of note since this metabolite is thought to be linked to diclofenac hepatotoxicity (*57-61*), and as such may be of interest in relation to current understanding as to why diclofenac is so toxic to *Gyps* sp. Further work in this area is certainly required.

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## Conclusion

These data demonstrate that once developed, an ELISA test of this type can provide a rapid, specific, reliable, and affordable alternative to otherwise costly and highly advanced techniques such as GC-MS and LC-ESI/MS.

It has been demonstrated that in all investigated WWTPs (n = 21), diclofenac was present in the effluents at an average concentration of approximately 1 µg/L. This provides further confirmation of the widespread distribution of diclofenac in the aquatic environment. Further, the biodegradation study revealed an average removal rate of only about 5%. Therefore, it appears that diclofenac is not significantly eliminated by either biodegradation or sorption. Further investigations are however necessary in order to elucidate the formation and stability of diclofenac metabolites in WWTPs and receiving surface waters, and in order to fully understand the effect of photodegradation of these compounds in these systems.

In applying this technique to tissue extracts, it has been demonstrated that comparable data can be produced with almost the same detection limit (as LC-ESI/MS) in what are, by nature, quite matrix complex and highly variable extracts of a range of animal tissues. Globally, conservation projects commonly work under restricted budgets, and in the developing world this is even more likely to be the case. Plans are now in place to utilize this affordable ELISA methodology on-site within vulture sanctuaries in India which are directly engaged in important Critically Endangered vulture breeding programs.

Undoubtedly, there is also a great deal of scope for further application of this and many other similar ELISA's, as interest amongst scientists grows regarding the wider understanding of the fate and behaviour of an ever increasing range of 'new' and poorly understood micropollutants.

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## Chapter 14

# A Comprehensive Approach to Biological Monitoring of Pesticides in Urine

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In the past, we have had almost limitless urine for biological measurements but this has changed dramatically as the study populations continue to focus on young children for whom urine collection is difficult and as the number of pesticides for which exposure information is needed has increased. In order to accommodate the biological monitoring component of these studies, we have refined our methods to allow maximum exposure information from a limited-volume urine sample. Using three separate analytical methods, each requiring only 2 mL of urine, we can successfully measure 35 different pesticides or metabolites at background levels with a high degree of selectivity and precision. We describe a comprehensive approach to biological monitoring of current-use pesticides in urine using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and gas chromatography-MS/MS using isotope dilution.

U.S. government work. Published 2007 American Chemical Society.

In 1999, an estimated 415,000 tons of conventional pesticides were applied in the United States *(1)*. Biological monitoring is a useful tool for establishing the presence and magnitude of pesticide exposures *(2)*. Many of the methods available in the literature focus on specific pesticide classes or individual pesticides or metabolites *(3-11)*. Few methods are available for the analysis of markers of several classes of pesticides in the same sample in the low ng/mL range, which is generally necessary for studies of non-occupationally exposed individuals. However, in epidemiological studies, it is often important to measure biomarkers of many different pesticides to get an accurate representation of an individual's exposure in order to make adequate statistical interpretations of health outcome data. We have accomplished this in our laboratory by analyzing small aliquots of the same urine sample using a number of different methods *(8;11;12)*. This has allowed us to obtain biologically-based data on 35 different chemicals using approximately 6 mL of urine – an amount of urine that can easily be obtained from adults and children. The target analytes for our methods represent pesticides from several different pesticide classes--organophosphorus insecticides, synthetic pyrethroid insecticides, triazine herbicides, chloroacetanilide herbicides, phenoxyacetic acid herbicides, carbamates, fungicides and fumigants, as well as the topical insect repellant *N,N*-diethyl-*m*-toluamide (Table I).

We describe a comprehensive approach to biological monitoring of current-use pesticides in urine using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and gas chromatography-MS/MS with quantification using isotope dilution. Using three separate analytical methods, each requiring only 2 mL of urine, we can successfully measure 35 different pesticides or metabolites at background levels with a high degree of selectivity and precision. The methods we have developed are diverse in character but all use selective detection techniques and isotope dilution quantification. Our methods are precise, reliable and robust with low limits of detection (LOD).

## Materials and Methods

### Chemicals

All chemicals used were of analytical grade. 1-Chloro-3-iodopropane and β-glucuronidase from *Helix pomatia* (G 0751, EC 3.2.1.31, type H-1) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The native standards of acetochlor mercapturate; N-acetyl-S-[2-(2-ethyl-6-methylphenyl)(ethoxymethyl)amino]-2-oxoethyl-L-cysteine (ACE), alachlor mercapturate; N-acetyl-S-[2-(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoethyl-L-cysteine (ALA), metolachlor mercapturate; N-acetyl-S-[2-(2-ethyl-6-

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**Table I. The target analytes, abbreviations, their metabolic status, and parent pesticide**

| *Analyte name* | *Abbreviation* | *Indicator of Exposure to (Pesticide Class [a])* |
|---|---|---|
| Atrazine mercapturate; C | ATZ | Atrazine (TAH) |
| Acetochlor mercapturate;C | ACE | Acetochlor (CAH) |
| Metolachlor mercapturate; C | MET | Metolachlor (CAH) |
| Alachlor mercapturate;C | ALA | Alachlor (CAH) |
| 2,4,5-Trichlorophenoxyacetic acid; P | 2,4,5-T | 2,4,5-T (PH) |
| 2,4-Dichlorophenoxyacetic acid; P | 2,4-D | 2,4-D (PH) |
| 4-Nitrophenol; M | PNP | Parathion * (OPI) |
| 5-Chloro-1-isopropyl-[3H]-1,2,4-triazol-3-one; M | CIT | Isazophos * (OPI) |
| 3-Chloro-4-methyl-7-hydroxycoumarin; M | CMHC | Coumaphos * (OPI) |
| 2-Diethylamino-6-methyl pyrimidin-4-ol; M | DEAMPY | Pirimiphos * (OPI) |
| 2-[(dimethoxyphosphorothioyl)sulfanyl] succinic acid; M | MDA | Malathion * (OPI) |
| 3,5,6-Trichloro-2-pyridinol; M | TCPY | Chlorpyriphos * (OPI) |
| 2-Isopropyl-6-methyl-4-pyrimidiol; M | IMPY | Diazinon * (OPI) |
| Dimethylphosphate; M | DMP | O,O-Dimethyl substituted organophosphorus pesticides (OPI) |
| Dimethylthiophosphate; M | DMTP | O,O-Dimethyl substituted thio organophosphorus pesticides (OPI) |

*Continued on next page.*

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Table I. *Continued.*

| *Analyte name* | *Abbreviation* | *Indicator of Exposure to (Pesticide Class [a])* |
|---|---|---|
| Dimethyldithiophosphate; M | DMDTP | O,O-Dimethyl substituted dithio organophosphorus pesticides (OPI) |
| Diethylphosphate; M | DEP | O,O-Diethyl substituted organophosphorus pesticides (OPI) |
| Diethylthiophosphate; M | DETP | O,O-Diethyl substituted thio organophosphorus pesticides (OPI) |
| Diethyldithiophosphate; M | DEDTP | O,O-Diethyl substituted dithio organophosphorus pesticides (OPI) |
| 4-Fluoro-3-phenoxy benzoic acid; M | 4-F-3-PBA | Cyfluthrin (PI) |
| 3-Phenoxy benzoic acid; M | 3-PBA | 10 of 18 Commercially available pyrethroids in the US (PI) |
| *cis*-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; M | DBCA | Deltamethrin (PI) |
| *cis and trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acids; M | *cis-/ trans-* DCCA | Cyfluthrin, permethrin, cypermethrin (PI) |
| *N,N*-Diethyl-*m*-toluamide; P | DEET | DEET (IR) |
| 2-Isopropoxyphenol; M | IPP | Propoxur (CI) |

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| | | |
|---|---|---|
| 2,3-Dihydro-2,2-dimethyl-7-hydroxybenzofuran (Carbofuranphenol); M | CFP | Carbofuran, benfuracarb, carbosulfan, furathiocarb (CI) |
| 1-Naphthol; M | 1N | Carbaryl (CI); naphthalene (FG, PAH) |
| 2-Naphthol; M | 2N | Naphthalene (FG, PAH) |
| *o*-Phenylphenol; P | OPP | OPP (F) |
| 2,5-Dichlorophenol; M | 25DCP | *p*-Dichlorobenzene (FG, CH) |
| 2,4-Dichlorophenol; M | 24DCP | *m*-Dichlorobenzene (CH) |
| 2,4,5-Trichlorophenol; M/P | 245TCP | 245TCP, trichlorobenzene, pentachlorophenol, lindane (CH) |
| 2,4,6-Trichlorophenol; M/P | 246TCP | 246TCP, trichlorobenzene, pentachlorophenol, lindane (CH) |
| Pentachlorophenol; P | PCP | Pentachlorophenol (F) |

[a] TAH = triazine herbicides, CAH = chloroacetoanilides herbicides, CI = carbamate insecticide; CH* = Chlorinated hydrocarbon; F = fungicide; FG = fumigant; PAH = polycyclic aromatic hydrocarbon; PH = phenoxyacetic acid herbicides, OPI = organophosphate insecticides, PI = pyrethroid insecticides, IR = insect repellant, C = conjugate; P = parent pesticide; M = metabolite.

*or their methyl counterparts

methylphenyl)(2-methoxy-1-methylethyl)amino]-2-oxoethyl-L-cysteine (MET), 5-chloro-1,2-dihydro-1-isopropyl-[3H]-1,2,4-triazol-3-one (CIT), and 3-chloro-4-methyl-7-hydroxycoumarin (CMHC) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA). The 2-diethylamino-6-methyl pyrimidin-4-ol (DEAMPY), 2-isopropyl-6-methyl-4-pyrimidiol (IMPY), 3,5,6-trichloro-2-pyridinol (TCPY) and *N,N*-diethyl-*m*-toluamide (DEET), carbofuranphenol (CFP) and diethylphosphate (DEP) (98%) were purchased from Chem Services (West Chester, PA, USA). The 2-[(dimethoxyphosphorothioyl)sulfanyl]succinic acid (MDA) was obtained from Cheminova Agro A/S (Lemvig, Denmark) and the atrazine mercapturate; N-acetyl-S-[4-(ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl]-L-cysteine (ATZ) was a gift from the University of California at Davis. The 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-isopropoxyphenol (IPP), 2, 5-dichlorophenol (25DCP), 2, 4-dichlorophenol (24DCP), 2, 4, 5-trichlorophenol (245TCP), *ortho*-phenyl phenol (OPP), pentachlorophenol (PCP), 4-nitrophenol (PNP), diethylthiophosphate (DETP) (98%) and diethyldithiophosphate (DEDTP) (97%) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). 2, 4, 6-Trichlorophenol (246TCP) was obtained from Eastman Kodak Co. (Rochester, NY). 1-Naphthol (1N) and 2-naphthol (2N) were obtained from Janssen Chimica (Geel, Belgium). The *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acids (*cis*-DCCA, *trans*-DCCA) and the 4-fluoro-3-phenoxybenzoic acid (4F3PBA) were generous gifts from Dr. Jürgen Angerer, (Erlangen University, Erlangen, Germany). The *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DBCA), and 3-phenoxybenzoic acid (3PBA) were purchased from EQ Laboratories (Augsburg, Germany). Dimethyl phosphate (DMP) (33%) was purchased from AccuStandard Inc (New Haven, CT). The impurities in DMP primarily consisted of the O-monoalkyl substituted phosphate and inorganic phosphate, compounds which did not hinder our analysis. Native standards of dimethylthiophosphate (DMTP) (98%) and dimethyldithiophosphate (DMDTP) (98%) were purchased from AppliChem (Darmstadt, Germany).

No further purifications were performed for the target analytes, but the purities were used to calculate the final concentration of the native analytes. All labeled standards were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA), except for the $D_7$-labeled MDA that was provided by the California State Department of Health Services (Berkeley, CA, USA) and the $^{13}C_6$ 1-naphthol which was synthesized in house at CDC. The labeled analytes were labeled with $^{13}C$, $^{15}N$, and/or $^{2}H$ with the patterns indicated in Tables II.

## Quality Control Materials

Urine was collected from multiple anonymous donors and divided into three pools. The first quality control pool (low concentration) was spiked with the

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native materials to yield an approximate analyte concentration of 5-10 µg/L (QCL). The second quality control pool (high concentration) was spiked with the native materials to yield an approximate analyte concentration of 15-20 µg/L (QCH). The third pool was not spiked (blank urine). This pool was used as matrix material for calibration standards and blanks.

## Standard Preparation

### *Labeled Standards*

Approximately 0.5 mg of each labeled standard were mixed and diluted with acetonitrile to obtain a concentration of 20 µg/mL. This solution was further diluted to give an approximate concentration of the individual labeled compounds of 1 µg/mL. This solution was used as an insernal standard ISTD spiked (10 µL) in all unknown samples, quality control (QC) materials, and calibration standards.

### *Native Standards.*

Individual stock solutions (approximately 200 µg/mL in acetonitrile) of the unlabeled analytes were prepared from pure substance. From the stock solutions adequate volumes were taken by pipette to prepare eight calibration standard solutions with the following concentrations of the individual analytes; 0.020, 0.040, 0.080, 0.20, 0.40, 0.80, 2.0, 4.0 µg/mL. To prepare a calibration curve 25 µL of each standard solution was added to each 2 mL blank urine samples.

## Biological Samples

Most of the analytes were measured in urine samples collected in 1999 and 2000 as a part of the ongoing National Health and Nutrition Examination Survey (NHANES). The survey has been described in more detail elsewhere *(13)*. Urine samples were frozen within 4 h of collection and were stored at –20 °C prior to analysis. The samples tested represented a random one-third subset of the urine samples collected from participants aged 6 to 59 years. Because of the random selection of the subset, the representativeness of the survey was maintained. Subset-specific sample weights were applied to the data as previously described *(14)*. SAS (SAS Institute, Cary, NC) and SUDAAN (RTI International, Research Triangle Park, NC) software were used for data analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects.

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**Table II. Optimized precursor/product ion pairs, declustering potential (DP), collision cell exit potential (CXP), and the collision offset energy (CE) for the multi-analyte method target analytes on the Sciex API4000 and TSQ 7000 mass spectrometers.**

| *Analyte (label pattern)* | *Precursor ion* | | *Product ion* | | | *Ion Mode* | *DP (V)* | *CE (V)* | *CXP (V)* |
|---|---|---|---|---|---|---|---|---|---|
| | *Native* | *Labeled* | *Native* | *Labeled* | *Instrument* | | | | |
| *Trans*-DCCA ($^{13}C_2$) | 207 | 210 | 35 | 35 | Sciex | - | -50 | 30 | -5 |
| *cis*-DCCA[a] | 207 | [a] | 35 | [a] | Sciex | - | -50 | 30 | -5 |
| DBCA[a] | 294 | [a] | 79 | [a] | Sciex | - | -35 | 18 | -3 |
| 3PBA ($^{13}C_6$) | 213 | 219 | 93 | 99 | Sciex | - | -55 | 28 | -7 |
| 4F3PBA[b] | 231 | [b] | 93 | [b] | Sciex | - | -60 | 36 | -17 |
| IMPY ($^{13}C_4$) | 153 | 157 | 84 | 88 | TSQ | + | NA | -22 | NA |
| DEAMPY ($d_6$) | 182 | 188 | 154 | 158 | TSQ | + | NA | -22 | NA |
| CIT ($d_7$) | 203 | 210 | 120 | 121 | TSQ | + | NA | -24 | NA |
| ATZ ($^{13}C_3$) | 343 | 346 | 214 | 217 | TSQ | + | NA | -23 | NA |
| ACE ($^{13}C_6$) | 351 | 357 | 130 | 130 | TSQ | + | NA | -15 | NA |
| ALA ($^{13}C_6$) | 365 | 371 | 162 | 168 | TSQ | + | NA | -25 | NA |
| DEET ($d_6$) | 192 | 198 | 119 | 119 | TSQ | + | NA | -22 | NA |
| MDA ($d_7$) | 273 | 280 | 141 | 147 | TSQ | - | NA | 13 | NA |
| PNP ($^{13}C_6$) | 138 | 144 | 108 | 114 | TSQ | - | NA | 22 | NA |
| CMHC ($^{13}C_4$) | 209 | 213 | 145 | 148 | TSQ | - | NA | 24 | NA |
| MET ($^{13}C_6$) | 409 | 415 | 280 | 286 | TSQ | - | NA | 21 | NA |
| 2,4-D ($^{13}C_6$) | 219 | 225 | 161 | 167 | TSQ | - | NA | 18 | NA |
| TCPY 2[c] ($^{13}C_5{}^{15}N$) | 196 | 202 | 196 | 202 | TSQ | - | NA | 15 | NA |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| TCPY 1 ($^{13}C_5{}^{15}N$) | 198 | 204 | 198 | 204 | TSQ | - | NA | 15 | NA |
| 2,4,5-T ($^{13}C_6$) | 255 | 261 | 197 | 203 | TSQ | - | NA | 16 | NA |
| 3-PBA ($^{13}C_6$) | 213 | 219 | 93 | 99 | TSQ | - | NA | 25 | NA |
| DMP ($d_6$) | 203 | 209 | 127 | 133 | TSQ | + | NA | -12 | NA |
| DMTP ($d_{10}$) | 219 | 225 | 143 | 149 | TSQ | + | NA | -13 | NA |
| DMDTP ($d_6$) | 235 | 241 | 125 | 131 | TSQ | + | NA | -10 | NA |
| DEP ($d_6$) | 231 | 241 | 127 | 133 | TSQ | + | NA | -13 | NA |
| DETP ($d_{10}$) | 247 | 257 | 191 | 193 | TSQ | + | NA | -12 | NA |
| DEDTP ($^{13}C_4$) | 263 | 267 | 153 | 157 | TSQ | + | NA | -12 | NA |
| IPP [d] | 229 | 235 | 187 | 193 | TSQ | + | NA | 5.9 | NA |
| 25DCP ($^{13}C_6$) | 239 | 245 | 163 | 169 | TSQ | + | NA | 7.2 | NA |
| 24DCP ($^{13}C_6$) | 239 | 245 | 163 | 169 | TSQ | + | NA | 7.2 | NA |
| CFP [e] | 247 | 247 | 199 | 205 | TSQ | + | NA | 7.2 | NA |
| 246TCP ($^{13}C_6$) | 273 | 279 | 197 | 203 | TSQ | + | NA | 7.5 | NA |
| TCPY ($^{13}C_5{}^{15}N$) | 274 | 280 | 198 | 204 | TSQ | + | NA | 7.5 | NA |
| PNP ($^{13}C_6$) | 216 | 219 | 140 | 143 | TSQ | + | NA | 10.1 | NA |
| 245TCP ($^{13}C_6$) | 273 | 279 | 197 | 203 | TSQ | + | NA | 7.5 | NA |
| 1N ($^{13}C_6$) | 221 | 227 | 145 | 151 | TSQ | + | NA | 10.1 | NA |
| 2N [f] | 221 | 227 | 145 | 151 | TSQ | + | NA | 10.1 | NA |
| OPP ($^{13}C_6$) | 247 | 253 | 171 | 177 | TSQ | + | NA | 13 | NA |
| PCP [g] ($^{13}C_6$) | 228 | 234 | NA | NA | TSQ | - | NA | NA | NA |

[a] labeled *trans*-DCCA used as internal standard

[b] labeled 3-PBA used as internal standard

[c] confirmation ion

[d] = labeled 25DCP used as internal standard

[e] = labeled 246TCP used as internal standard

[f] = labeled 1N used as internal standard

[g] = MS analysis only

NA = parameter not applicable to instrument used

## Sample Preparation

### *Multi-class Method*

Aliquots (2 mL) of urine were spiked with 25 µL isotopically labeled internal standards giving an approximate 25 ng/mL concentration of the standards in the urine *(12)*. In order to hydrolyze possible glucuronide or sulfate conjugated metabolites, β-glucuronidase type H-1 from *Helix pomatia*, with a specific activity of approximately 500 units/mg, was used. To each sample, an amount of enzyme giving 800 units of activity dissolved in 1.5 mL of a 0.2 M acetate buffer (3.1 mL glacial acetic acid, 9.7 g sodium acetate, 1 L water) was added. The samples were incubated for 17 h at 37 °C and then extracted using solid phase extraction (SPE). The SPE cartridges (Oasis HLB 3cc, Waters, Milford, MA, USA) were preconditioned with 1 mL of methanol followed by 1 mL of 1% acetic acid. The samples were added and passed through the cartridges. The cartridges were washed with a 5% methanol in 1% acetic acid solution (1 mL). The cartridges were dried for approximately 30 s using vacuum. Methanol (1.5 mL) was eluted through the cartridges and collected. Two mL of acetonitrile was added to the methanol fractions and the combined extracts were evaporated to dryness using a Turbovap LV (Zymark, Hopkinton, MA) at 40 °C and 10 psi of nitrogen as the evaporating gas. The dried residues were reconstituted in 50 µL acetonitrile. The samples were separated into two fractions, 10 µL and 40 µL, for analysis using HPLC-TSI-MS/MS and HPLC-APCI-MS/MS, respectively.

### *Dialkylphosphate Method*

Aliquots (2mL) of urine were pipetted into 15-mL tubes and spiked with 10 µL of the ISTD solution to give urinary ISTD concentrations of 25 µg/L for each analyte *(8;15)*. After the samples were mixed, they were placed in a commercial lyophilizer system (Labconco, Kansas City, MO). The lyophilizer was operated overnight in the program mode without further manual manipulation. For a run of 50 samples, the samples were initially frozen for 4 h at –34 °C and atmospheric pressure. After the samples were sufficiently frozen, the vacuum was set to 25.5 mTorr and the samples remained at –34 °C for an additional 4 h. The samples were then taken to –20 °C for 2 h, 0 °C for 1 h, and finally 20 °C for 1 h. The following day after completion of the lyophilization process, 2.0 mL acetonitrile and 2.0 mL ethyl ether were added to the residue in each sample tube, then vortex mixed for about 1 min. The supernatants were then poured into different 15-mL centrifuge tubes to separate them from the undissolved residue. The extraction tubes with the undissolved residue were rinsed with another 1 mL acetonitrile, vortex mixed, and combined with the supernatants. The samples were concentrated to approximately 1 mL in about 10 min using a Turbovap LV at 30 °C and 10 psi of nitrogen. The concentrated samples were poured into a 15-mL test tube, which contained a few

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grains of potassium carbonate. CIP (50 µL) was added to the samples and mixed. The samples were then placed in a dry bath set at 60 °C for 3 h. The supernatants were transferred to clean tubes and evaporated to dryness. The samples were reconstituted using 75 µL of toluene and analyzed using GC-MS/MS.

### *Phenols Method*

Urine samples (2.0 mL) were pipetted into 15-mL tubes and spiked with 5 µL of the ISTD solution to give an approximate 25 µg/L concentration of the internal standard in the urine *(11)*. To hydrolyze possible glucuronide- or sulfate-bound metabolites, β-glucuronidase (120,000 units/sample) in 2 mL 0.1 M acetate buffer (pH 4.5) was used. Samples were incubated for 17 h at 37 °C. The urine hydrolysates were extracted using 3cm$^3$ Oasis ® SPE cartridges that were preconditioned with 1 mL of 20% ethyl ether/n-butyl chloride, 1 mL methanol, and 1 mL 0.05N HCl. The urine hydrolysates were acidified with 250 µL 2M $H_2SO_4$ and then applied to the cartridges. The cartridges were washed with a 5% methanol solution and eluted with 4 mL of 20% ethyl ether/n-butyl chloride. To the eluates, 1 mL of 3N NaOH was added and vortex mixed to extract the analytes from the organic phase into the aqueous phase. The organic layers were discarded and the aqueous layers were collected and placed into new centrifuge tubes. The phenolic compounds were chemically derivatized to their respective chloropropyl ethers by adding 0.5 mL 0.4 M $TBAHSO_4$ and 0.5 mL 10% CIP in n-butyl chloride and incubating in a 60 °C drybath for 1 h. The reaction mixtures were cleaned using 3cm$^3$ ChemElut ® sorbent-immobilized liquid extraction 3cm$^3$ cartridges (Varian Sample Preparation Products, Walnut Creek, CA). The reaction mixtures were applied to the cartridges and eluted twice with 2 mL hexane. The samples were evaporated to dryness using a Turbovap LV Evaporator at 30 °C and 10 psi of nitrogen for approximately 30 min. Samples were reconstituted with 75 µL of toluene and analyzed using GC-MS/MS.

## Instrumental Analysis

All analyses were performed on a TSQ 7000 triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled to a GC or HPLC or a Sciex API4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) coupled to an HPLC. The high-performance liquid chromatography (HPLC) was performed on an Agilent 1100 system (Agilent Tech., Waldbronn, Germany) consisting of a binary pump, a degasser, an auto sampler, and a temperature-stable column compartment. For the atmospheric pressure chemical ionization (APCI) application, a TSQ 7000 was used. For the turbo ionspray atmospheric pressure ionization (TSI) application, the Sciex was

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used. The GC used was a TraceGC (ThermoQuest, San Jose, CA) with a split/splitless injector and a CTC A200s autosampler (Carrboro, NC).

### *Multi-analyte Method - HPLC Operating Conditions*

An isocratic elution with a mobile phase mixture of 36% acetonitrile in water with 0.1% acetic acid was used for the analysis on the TSQ 7000. The chromatographic separation was performed on a Betasil Phenyl column (5 µm particle size, 100A pore size and 4.6 mm I.D. x 100 mm length) from Keystone Scientific Inc. (Bellefonte, PA, USA). The flow rate was 1.0 mL/min and the injection volume was 10 µL. The column temperature was kept at 25 °C during the analysis.

For the analysis on the Sciex API4000, an isocratic elution was also used. The solvent mixture was 51% acetonitrile in water with 0.1% acetic acid. The column was a Betasil $C_{18}$ column (5 µm particle size, 100A pore size and 1mm I.D. x 100 mm length) from Keystone Scientific Inc. (Bellefonte, PA, USA). The flow rate was 0.05 mL/min and the injection volume was 2 µL. The column was kept at 35 °C.

### *Multi-analyte Method - Mass spectrometry Operating Conditions*

The Sciex API4000 was operated in the multiple reaction monitoring (MRM) mode using negative ion TSI. Zero air was used for collision-activated dissociation (CAD), nebulizer and heater gases. All other gases were nitrogen. Ion source, collision and curtain gases had optimum pressures of 16, 6 and 20 psi, respectively. The heater gas (450°C) was operated with a pressure of 16 psi. The ionspray current was -4.5 kV and the entrance potential was -10 V. Optimized filter parameters for the precursor / product ion pairs are listed in Table II. The samples were analyzed in negative ionization mode and all analytes were measured in one segment. The total runtime was less than 9 min.

The TSQ 7000 was operated with the heated capillary at 450 °C, the corona discharge at 4.0 kV and a capillary temperature of 250 °C. The sheath gas ($N_2$) pressure was set to 50 psi and the collision gas (Ar) pressure to 2 mT.

The precursor/product ion pairs as well as the collision offset energy used for the analysis of the different compounds are summarized in Table II. All samples were injected twice. For the first injection, data were acquired in positive ionization mode and the total runtime was 7.25 min. The positive run was divided into two distinct timed segments, 0-3.5 min and 3.5-7.25 min. For the second injection, data were acquired in the negative ionization mode and the total runtime was 13 min. Again, the run was divided into distinct timed segments. In total, five time segments were used: 0-3.2 min, 3.2-4.3 min, 4.3-6.8 min, 6.8-9 min, and 9-13 min.

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### *Dialkylphosphate Method - GC Conditions*

Samples (1 µL) were injected into the GC by automatic splitless injection with an injection purge delay of 60 s. The GC was coupled to a TSQ7000. A 30-m J & W (Folsom, CA) DB-5MS ([5% phenyl]-methyl polysiloxane, 0.25 µm film thickness, 0.25 mm id) capillary column was used for separation of the chloropropyl phosphate esters. A guard column (deactivated fused silica column, Restek, Bellefonto, PA) was used to help extend the useful life span of the analytical column. The temperatures of the injector and transfer line were 250 °C. The column temperature was initially 80 °C for 2 min and was then heated linearly to 250 °C at 17 °C/min. The final temperature of 250 °C was held for 2 min.

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### *Dialkylphosphate Method - Mass Spectrometry Operating Conditions*

The chloropropyl phosphate esters were analyzed using MRM with chemical ionization (CI) in the positive ion mode. Methane was used as a reagent gas with a pressure of 1500 mT and argon as a collision-induced dissociation gas with a pressure of 2 mT. A full auto-tune of the mass spectrometer was performed before analysis of every set of samples. MS conditions were as follows: source temperature was 150 °C, electron energy was 200 eV, and the potential for the continuous dynode electron multiplier varied depending upon multiplier lifetime. Table II summarizes the characteristic precursor/product ion combinations and collision offsets used in measuring each analyte and ISTD with a width mass window of 0.4 amu and a scan rate of 0.03 $s^{-1}$. The run was divided into distinct timed segments. In total, five time segments were used: 7.0-7.5 min, 7.5-8.5 min, 8.5-9.2 min, 9.2-9.9 min, and 9.9-10.4 min. The product ions for each precursor ion were selected to maximize specificity, sensitivity, and linear dynamic range.

### *Phenols Method - GC Conditions*

Samples (1 µL) were injected into the gas chromatograph (TraceGC, ThermoQuest, San Jose, CA) by splitless injection using an autosampler (CTC A200s, Carrboro, NC) with an injection purge delay of 60 s. The GC was coupled to a triple quadrupole mass spectrometer (FinniganTSQ-7000, ThermoFinnigan, San Jose, CA). A 30-m J & W (Folsom, CA) DB-5MS ([5% phenyl]-methyl polysiloxane, 0.25 µm film thickness, 0.25 mm id) capillary column was used for separation of the chloropropyl phenol esters. A guard column (deactivated fused silica column, Restek, Bellefonto, PA) was used to help extend the useful life span of the analytical column. The temperatures of the injector and transfer line were 250 °C. The column temperature was initially 80 °C for 2 min and was then heated linearly using two ranges: to 160 °C at 10 °C/min and then to 260 °C at 4 °C/min. The final temperature of 260 °C was held for 2 min.

*Phenols Method - Mass spectrometry operating conditions*

The chloropropyl phenols ethers, except PCP, were analyzed using multiple reaction monitoring (MRM) with CI in the positive ion mode. PCP was analyzed using negative CI selected ion monitoring (SIM) MS was used. Methane was used as a reagent gas with a pressure of 1500 mT and argon as a collision induced dissociation gas with a pressure of 2 mT. A full auto-tune of the mass spectrometer was performed before analysis of every set of samples. MS conditions were as follows: source temperature was 150 °C, electron energy was 200 eV, and the potential for the continuous dynode electron multiplier varied depending upon multiplier lifetime. Table II summarizes the characteristic precursor/product ion combinations and collision offsets used in measuring each analyte and ISTD with a width mass window of 0.4 amu and a scan rate of $0.06S^{-1}$. In total, six time segments were used: 9.5-11 min, 11-12.8 min, 12.8-14 min, 14-15.4 min, 15.4-16.4 and 16.4-21 min. The product ions for each precursor ions were selected to maximize specificity, sensitivity, and linear dynamic range.

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## Data Processing

Peaks were automatically integrated using the Xcalibur® software (version 2.1, ThermoQuest, San Jose, CA) provided with the TSQ7000 or using Analyst® software (Applied Biosystems/MDX Sciex, Foster City, CA) provided with the Sciex API4000. The background signal was subtracted, and all data were smoothed. The analyst checked and corrected any discrepancies in peak selection to provide an accurate integration. Peak areas and other pertinent data associated with the analysis were exported into a Microsoft EXCEL® file and loaded into a Microsoft ACCESS® database for permanent storage. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

## Quantification and Quality Control of Analytical Runs

A seven or eight point calibration plot for quantification was made up for every analytical run using blank urine. The concentrations of the calibration standards ranged from 0.25 to 100 ng/mL for most analytes. A linear regression equation from the best fit line of a plot of the calibration standard concentration against the $\text{area}_{\text{native}}/\text{area}_{\text{ISTD}}$ was used to calculate analyte concentrations in individual samples. For each analytical run, seven or eight calibration samples, two fortified urine samples (one high and one low dose), one blank urine sample and one solvent blank, were prepared, extracted, and analyzed in parallel with the unknown samples. Quality control of the analytical runs was judged according to Westgard multirules for quality control *(16)*.

### Method Validation

All methods were validated to determine limits of detection (LOD; $3s_0$), extraction efficiency; precision an intra- and inter-method comparative values using the methods outlined previously (*11, 12, 15, 17*).

## Results

The method validation data (extraction recovery, relative standard deviations, and LODs) for the analytes are summarized in Table III. The recovery, LOD, and RSD results were similar though slightly better results were obtained using TSI. Furthermore, the results of urine samples analyzed with both TSI and APCI (n=550) showed a good agreement ($r^2$=0.9937) between the two measurements even though the ionization techniques differ.

A comparison of PNP and TCPY concentrations in 42 urine samples measured using both the multi-class and phenols methods also showed good agreement ($r^2$=0.9817 and 0.9815, respectively). The number of samples with detectable concentrations of PNP and TCPY was 26 and 21, respectively.

Distributions of selected pesticide metabolites are shown in Table IV *(14,18)*. Of the analytes measured, 25DCP, 24DCP, 1N, 2N, TCPY, DEP, DEDTP, DMP, DMTP, DMDTP, and OPP were present in greater than 50% of the samples tested. In fact, 25DCP and TCPY were the most frequently detected in 85% and 90% of the population, respectively. In general, children had higher concentrations than did adults.

## Discussion

Methods for collecting urine samples from infants and children are difficult and often burdensome to the participant. Several urine collection methods have been reported for infants including the use of urine collection bags and expressing urine from cotton inserts in diapers. These methods typically result in the collection of small volumes of urine which then would limit the number of analyses that can be performed on that sample. In order to maximize the information obtained from these small-volume collections, we have optimized our methods to use as little urine as possible while still maintaining suitable analytical parameters to allow the detection of urinary concentrations resulting from background exposures.

If all methods are run concurrently, 36 samples can be prepared, analyzed, and resulting data quantified for all 35 target analytes in about 36 h. However, less than 30 h of total analyst time is typically involved, with the bulk of the analyst time dedicated to the methods with more complex sample preparation techniques. A large portion of the total analysis time is dedicated to the 17-h incubation time

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**Table III. Analytical specifications of the target analytes.**

| *Method* | *Analyte* | *Extraction recovery* | | | | *RSD (%)* | | *LOD (ng/mL)* |
|---|---|---|---|---|---|---|---|---|
| | | *Low level*[a] | | *High level*[b] | | | | |
| | | *Mean %* | *N* | *Mean (%)* | *N* | *Low pool (N)* | *High pool (N)* | |
| Multi-class | MDA | 75± 6 | 19 | 68 ± 5 | 10 | 6 (25) | 5 (25) | 0.3 |
| | PNP | 95 ± 3 | 19 | 93 ± 4 | 10 | 6 (83) | 5 (83) | 0.1 |
| | CMHC | 95 ± 7 | 19 | 96 ± 13 | 10 | 10 (85) | 9 (82) | 0.2 |
| | MET | 91 ± 9 | 19 | N/A | NA | 9 (85) | 9 (81) | 0.2 |
| | 24D | 96 ± 9 | 19 | 87 ± 4 | 10 | 6 (82) | 6 (79) | 0.2 |
| | TCPY 1 | 88 ± 8 | 19 | 93 ± 9 | 10 | 9 (84) | 9 (81) | 0.3 |
| | TCPY 2 $^{C}$[c] | 93 ± 10 | 19 | 94 ± 8 | 10 | 9 (25) | 6 (25) | 0.4 |
| | 245T | 97 ± 5 | 19 | 90 ± 2 | 10 | 6 (85) | 5 (82) | 0.1 |
| | 3PBA (APCI) | 95 ± 5 | 19 | 90 ± 4 | 10 | 5 (25) | 5 (25) | 0.2 |
| | IMPY | 99 ± 12 | 19 | 81 ± 9 | 10 | 11 (83) | 10 (82) | 0.7 |
| | DEAMPY | 98 ± 7 | 19 | 95 ± 3 | 10 | 9 (80) | 8 (82) | 0.2 |
| | CIT | 98 ± 20 | 19 | 90 ± 11 | 10 | 14 (25) | 12 (25) | 1.5 |
| | ATZ | 96 ± 4 | 19 | 94 ± 2 | 10 | 8 (82) | 6 (80) | 0.3 |
| | ACE | 98 ± 5 | 19 | 94 ± 3 | 10 | 8 (81) | 7 (79) | 0.1 |
| | DEET | 96 ± 4 | 19 | 93 ± 3 | 10 | 8 (83) | 8 (82) | 0.1 |
| | 3PBA (TSI) | 94 ± 4 | 19 | 92 ± 2 | 10 | 7 (86) | 5 (84) | 0.1 |
| | 4F3PBA | 106 ± 13 | 19 | 104 ± 9 | 10 | 7 (87) | 6 (87) | 0.2 |
| | *cis*-DCCA | 108 ± 15 | 19 | 101 ± 15 | 10 | 14 (87) | 10 (86) | 0.2 |
| | *trans*-DCCA | 95 ± 4 | 19 | 92 ± 2 | 10 | 7 (87) | 5 (86) | 0.4 |
| | DBCA | 114 ± 18 | 19 | N/A | NA | 15 (87) | 15 (87) | 0.1 |
| Dialkyl phosphate | DMP | 94 ± 8 | 6 | 95 ± 10 | 6 | 15 (84) | 11 (83) | 0.6 |
| | DEP | 99 ± 4 | 6 | 99 ± 4 | 6 | 13 (84) | 10 (83) | 0.2 |
| | DMTP | 100 ± 11 | 6 | 82 ± 10 | 6 | 13 (84) | 10 (83) | 0.2 |
| | DMDTP | 100 ± 4 | 6 | 82 ± 11 | 6 | 15 (84) | 14 (83) | 0.1 |
| | DETP | 82 ± 6 | 6 | 87 ± 9 | 6 | 15 (84) | 14 (83) | 0.1 |
| | DEDTP | 75 ± 3 | 6 | 85 ± 3 | 6 | 13 (84) | 11 (83) | 0.1 |
| Phenols | IPP | 84 ± 9 | 3 | 89 ± 9 | 3 | 13 (83) | 12 (83) | 0.4 |
| | 25DCP | 93 ± 3 | 3 | 94 ± 1 | 3 | 14 (83) | 10 (83) | 0.1 |
| | 24DCP | 94 ± 3 | 3 | 92 ± 2 | 3 | 10 (83) | 12 (83) | 0.3 |
| | CFP | 92± 7 | 3 | 95 ± 6 | 3 | 11 (84) | 11 (84) | 0.4 |
| | TCPY | 95 ± 3 | 3 | 94 ± 2 | 3 | 10 (83) | 9 (83) | 0.4 |
| | 245TCP | 80 ± 2 | 3 | 84 ± 2 | 3 | 17 (84) | 14 (83) | 0.9 |
| | 246TCP | 91 ± 6 | 3 | 95 ± 3 | 3 | 13 (83) | 14 (83) | 1.3 |
| | PNP | 97 ± 3 | 3 | 94 ± 3 | 3 | 8 (83) | 9 (83) | 0.8 |
| | 1N | 88 ± 2 | 3 | 93 ± 4 | 3 | 11 (84) | 9 (84) | 0.3 |
| | 2N | 97 ± 3 | 3 | 99 ± 4 | 3 | 11 (83) | 10 (83) | 0.2 |
| | OPP | 94 ± 5 | 3 | 93 ± 2 | 3 | 10 (84) | 8 (84) | 0.3 |
| | PCP | 64 ± 3 | 3 | 66 ± 1 | 3 | 10 (84) | 8 (84) | 0.5 |

[a] 5 ng/mL for multi-class method; 10 ng/mL for dialkylphosphate method; 25 ng/mL for phenols method; [b] 50 ng/mL for multi-class and dialkylphosphate methods; 100 ng/mL for phenols method; [c] confirmation ion; N = number of samples tested; RSD = relative standard deviation; LOD = limit of detection

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**Table IV. Distribution percentiles of selected target analytes in the U.S. population. Concentrations are expressed as ng/mL with creatinine-adjusted concentrations (μg/g creatinine) in parentheses.**

| *Analyte* | *Age Group (years)* | *N* | *Geometric Mean* | *Median* | *95th percentile* | *Frequency of detection* |
|---|---|---|---|---|---|---|
| 25DCP | All | 1989 | 6.01 (5.38) | 6.50 (5.60) | 440 (299) | 85 |
| 24DCP | All | 1990 | 1.11 (0.994) | 0.75 (0.794) | 22.0 (13.9) | 84 |
| OPP | All | 1991 | 0.494 (0.441) | 0.490 (0.413) | 2.00 (2.93) | 70 |
| TCPY | All | 1994 | 1.77 (1.58) | 1.70 (1.47) | 9.90 (8.42) | 91 |
| 24D | All | 1977 | * | <LOD | <LOD | 55 |
| DMP | All | 1949 | * | 0.740 (0.806) | 13.0 (16.1) | 53 |
| DEP | All | 1949 | 1.03 (0.924) | 1.20 (0.924) | 13.0 (12.1) | 70 |
| DMTP | All | 1948 | 1.82 (1.64) | 2.70 (2.12) | 46.0 (51.0) | 64 |

* It cannot be reliably calculated because frequency of detection < 60%; <LOD = less than limit of detection

that is necessary to deconjugate glucuronide- and sulfate-bound conjugates of many of the target analytes in the phenols and multi-class methods. A similar amount of time is required for the lyophilization step in the dialkylphosphate method.

We measured these analytes in urine samples from the general U.S. population to determine the suitability of our methods for measuring these analytes in persons with no known exposures to pesticides and to provide background concentrations, or reference ranges, for comparison to other populations. We frequently detected many of the target analytes indicating widespread exposure to the parent chemicals.

## Conclusions

We have demonstrated a comprehensive approach to biological monitoring of exposure to pesticides in urine samples that can be used even when the sample volume that is available is limited. Though our approach requires multiple methods and a substantial amount of personnel and instrument time, measurements of urinary concentrations of 35 different analytes are obtained potentially representing over 50 different pesticides. Our methods provide LODs suitable for detecting background exposures with good precision. Our approach will be useful in studies where limited amount of urine may be available for analysis such as in studies involving infants or small children.

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## Chapter 15

# Application of a Cyclodiene-Specific ELISA to Residue Monitoring of Agricultural Produce and the Environment in Vietnam

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To enhance monitoring capacity for pesticide residues in agricultural produce and environmental samples in Vietnam, a simple and rapid immunoassay for cyclodienes was developed. The assay showed a good sensitivity for endosulfan with an $IC_{50}$ of 1-2μg/L. A wide cross reactivity for its metabolites and other cyclodienes such as aldrin, endrin, dieldrin, heptachlor led to formatting an ELISA (Enzyme-Linked ImmunoSorbent Assay) kit with a broad specificity for cyclodienes. A simple sample preparation protocol also was developed for diverse

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samples including water, soil, vegetables and fruits. During the years 2002-2003 the cyclodiene ELISA kits were supplied to various research institutions in South and North Vietnam to collaboratively monitor the pesticide residues in water, soil, vegetable and fruit in the North, South and Central Vietnam. A total of 450 samples of vegetable and grapes were screened for cyclodiene residues using the ELISA. The validation of 10% of positive samples by gas chromatography (GC) method confirmed that the results of ELISA correlated well and were reliable.

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## Introduction

Cyclodienes are widely applied organochlorine pesticides which have been used on many different crops. Endosulfan, a mixture of α- and β-isomers, is an important compound of this group, allowed for agricultural use until 2005. The use of endosulfan has been restricted by Plant Protection Department of Vietnam, but its residues are still frequently found in vegetables and fruit. Regional surveys of pesticide usage indicate that endosulfan is utilized frequently on vineyards in Ninh Thuan, Vietnam. Residues of other cyclodienes have also been detected in crops, because of their stability and long half-life, even though their use in agriculture is now banned. Therefore, it is necessary to monitor the residues of cyclodiene pesticides in agricultural produce in Vietnam.

Instrumental techniques utilising gas chromatography (GC) and high-performance liquid chromatography (HPLC) are usually performed to analyze insecticidal contamination in water, soil and foods. These techniques are reliable, but their use for screening purposes has limitations such as cost, time of analysis, the requirement for sample extraction and clean up, and inability to perform tests in the field. These problems can be overcome by using ELISA (*1-4*). Immunoassay is a simple, rapid and cost-effective method for analysis with high throughput capacity and portability for on-site analysis. Using Lee *et al.*'s approach (5), the Southern Institute of Agricultural Engineering and Post-harvest Technology (SIAEP HCMC) has developed an ELISA kit capable of detecting cyclodiene pesticides in agricultural and environmental samples. Using the ELISA kits, vegetable, fruit, water and soil samples collected in North, Central and South Vietnam were screened for cyclodiene contamination. To evaluate the validity of ELISA results, positive samples were confirmed by GC. This paper describes a successful case of technical capacity building for Vietnam that combined the analytical power of both GC and ELISA to enhance food and environmental safety.

# Materials and Methods

## Reagents

Maxisorp™ microtiter plates were purchased from Nunc (Denmark). Horseradish peroxidase (HRP), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), Freund's complete and incomplete adjuvant, 3,3',5,5'-tetramethybenzidine (TMB), goat anti-rabbit IgG-HRP, protein A-sepharose and dialysis tubing were obtained from Sigma (St Louis, MO). *N*-Hydroxysuccinimide (NHS), *N,N*-Dicyclohexyl carbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), Tween 20, dichloromethane and dimethyl formamide (DMF) were purchased from Merck (Darmstadt, Germany). All other chemicals were either reagent or analytical grade. An ELISA reader (ELX 800-Lionheart) was used for the absorbance measurement of the microtitre plate. GC-ECD (Schimadzu GC 17A- $^{63}$Ni-ECD, column: Alltech BPX50, 30mx0.32mm) was used for the GC-analysis.

## Specific Methods

Hapten synthesis and conjugation to proteins and enzyme were carried out at the University of Sydney and antibody production and immunoassay development were carried at the SIAEP in Ho Chi Minh City.

### *Synthesis of Haptens (Figure 1)*

***(CYC -II):*** *4-Oxobutanoic acid, (4-(4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) succinimidyl ester*

The CYC-II hapten was synthesized using the method described by Stanker *et al.* (*9*) and Lee *et al.* (*5*). Briefly, 1-hydroxy-chlordene was prepared by the oxidation of chlordene, a Diels-Alder adduct of cyclopentadiene and hexachlorocyclopentadiene with $SeO_2$. It was then reacted with succinic anhydride to produce a hemissucinate.

***(CYC-IV):*** *6-(1,4,5,6,7,7-Hexachlorobicyclo[2,2,1]-heptane-2,3b-succinimido) caproic acid*

The CYC-IV hapten was synthesized using the method described by Brummel *et al.* (*10*). Briefly, chlordendic anhydride was treated with 6-aminocaproic acid in the presence of triethylamine.

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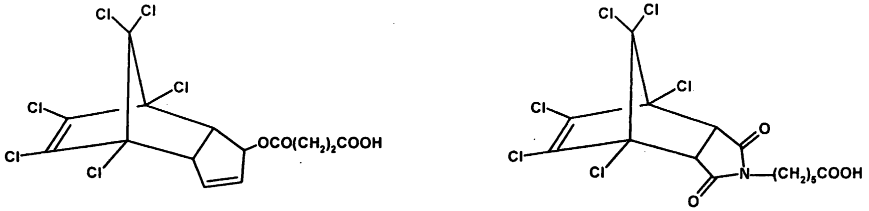

**CYC II** **CYC IV**

*Figure 1. Synthesized haptens for endosulfan immunoassay*

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### *Synthesis of NHS-Ester of Haptens*

*N*-Hydroxysuccinimide activated ester of each hapten was synthesized using the method described by Lee *et al.* (*5*). The acid was dissolved in dry dichloromethane under nitrogen. NHS, DCC and DMAP were added to the acid solution. The mixture was kept under stirring and allowed to react at room temperature overnight. After removing dicyclohexyl urea precipitates, the mixture was concentrated and the active ester was purified by silica gel flash chromatography.

### *Conjugation to Proteins*

The NHS esters of haptens were conjugated to proteins or HRP using the modified method of Lee *et al.* (*5*). Briefly, the NHS-ester dissolved in DMF was added slowly with stirring to a precooled protein solution. The reaction mix was allowed to stand at 4°C overnight, then was dialysed with PBS (50mM phosphate buffer + 0.9%NaCl).

### *Antibody Production*

Antibodies were raised by intradermal and intramuscular injections of CYC-II conjugated to keyhole limpet hemocyanin (KLH) into rabbits. The immunogen was diluted in 0.9% saline (1mg/mL) and emulsified in an equal volume of Freund's complete (first immunization) or incomplete adjuvant (subsequent immunization). After initial injection, a booster injection was given at monthly interval for six months. After the third injection, a small amount of blood was collected from the ear vein to check for the specific antibody (see below for procedure). Two weeks after the third injection, the antiserum was collected and purified by Protein A-sepharose affinity chromatography.

### *Indirect ELISA to Checking Specific Antibody Production*

First, the CYCII-BSA conjugate was coated using the carbonate coating buffer (pH 9.6) overnight at the concentration of 0.5µg/well. BSA was used to coat blank wells. Antiserum was diluted serially in PBS and added to the antigen-coated wells. After 30 min incubation, the wells were washed 3 times with PBS-T (PBS + 0.05% Tween 20), then goat-anti-Rabbit IgG- HRP (100µl/well) was incubated to the testing wells for 30 min. Finally, the wells were washed and substrate (TMB/hydrogen peroxide) was added for color development. Titre of each antiserum was determined (*17*).

### *Direct Competitive ELISA Method*

The competitive immunoassay of an immobilized antibody format was applied for endosulfan immunoassay. The antibody was diluted in carbonate buffer (pH 9.6) to 10µg/mL. The antibody solution (100µL) was added to each microwell for immobilization. After 12 h, the plate was washed with PBS-T and the nonspecific binding was blocked with 1% BSA-PBS at 200 µL/well for 1h. Endosulfan calibration solution or sample extract (50 µl/well) and enzyme conjugate (50µl/well) were added to the antibody-coated microwells and the mixture was incubated for 30 min. The optimised enzyme conjugate concentration was the lowest concentration that produces $A_{max}$ = 0.8 -1.4. After the incubation, the plate was washed and the color was developed by the incubating with 100 µL substrate mixture per well for 30 min. Color development was then stopped by addition of 50 µL 10% sulfuric acid per well and absorbance was read at 450 nm. Calculation of the inhibition is as follows:

$$\%Inhibition = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}}\right] \times 100$$

A: Absorbance; $A_{Control}$: Absorbance of negative sample; $A_{Blank}$: Absorbance of blank well

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### *GC Method for Endosulfan*

Vegetable sample (25g) in 70 mL acetone was blended, then filtered through a suction filter, rinsing the blender and the filter with 30 mL acetone. The filtrate was was extracted with 70 mL dichloromethane and 3-5g sodium chloride using a separating funnel. The organic layer was dried with sodium sulfate and rotary evaporated to 3 mL. The concentrate was loaded onto a silica column (2 g silica pre wash with hexane), the column was rinsed with 5 mL hexane and eluted with toluene to the final volume of 10 mL for GC analysis. Samples were analyzed by a GC-ECD (Schimadzu GC 17A- $^{63}$Ni-ECD, column: Alltech BPX50, 30mx0.32mm). Nitrogen was used as carrier gas at a flow rate of 10 mL/min. The column temperature: 120°C for 1 min, then enhaced at 10 °C/min to 240°C, held 5 min, and at 20°C/min to 280 °C, held 7 min. The temperature of the injector was 290°C, and the detector temperature was 300°C. 1μl of sample was injected in the splitless mode.

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## Results and Discussion

### Characterization of the cyclodiene ELISA kit

The antisera were collected as described in the methods section and IgG purified using Protein-A affinity chromatography. For direct competitive immunoassay, the CYCIV-enzyme conjugate was optimized by titrating against the purified antibody. The sensitivity of immunoassays for endosulfan using the optimized conditions was screened. The average standard curve of endosulfan (Figure 2) shows inhibition of antibody binding at low parts per billions with an $IC_{50}$ at 1-2μg/L. This sensitivity is well below the recommended maximum residue limit of endosulfan of 0.5 to 2 ppm (depending on kinds of samples) by Codex (*18*).

To determine the specificity of the antibody raised against the hapten CYCII, the cross-reactions with the endosulfan metabolites and other organochlorine pesticides were examined. The results are summarized in Table I.

The affinity of the obtained antibody for β-endosulfan was 2 times higher than that for α-endosulfan. This antibody was particularly sensitive to endosulfan sulfate, the major toxic metabolite of endosulfan. Strong cross reactivity for aldrin, endrin, dieldrin, heptachlor and chlordane was also observed, suggesting the immunoassay to be cyclodiene-selective.

### *Effect of pH, ions and ionic strengths*

*The* endosulfan standard *curves* in phosphate buffers at pH 3.0, 5.0, 7.4, 9.0, 10.0 and 11.0 were evaluated for effects of pH. The color development was

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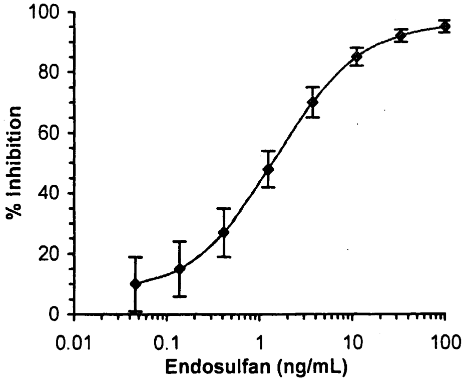

*Figure 2. Standard curve of endosulfan*

**Table I. Cross reactivity**

| *Compounds* | *$IC_{50}$ (μg/L)* | *Cross reactivity(%)* |
|---|---|---|
| Endosulfan technical (α:β=7:3) | 1.5 | 100 |
| α-Endosulfan | 1.5 | 100 |
| β-Endosulfan | 0.8 | 188 |
| Endosulfan sulfate | 0.6 | 250 |
| Endosulfan diol | 4.7 | 32 |
| Endosulfan lactone | 6.4 | 23 |
| Endosulfan hydroxy ether | 2.3 | 65 |
| Endosulfan ether | 1.0 | 150 |
| Aldrin | 0.6 | 250 |
| Dieldrin | 0.6 | 250 |
| Chlordane | 9.6 | 16 |
| Heptachlor | 3.1 | 48 |
| Endrin | 5.3 | 28 |
| Lindane | 68.7 | 2 |

$IC_{50}$: the concentration of analyte, which causes 50% inhibition of colour development not affected by pH and the assay sensitivity was only reduced at pH 11, probably due to hydrolysis.

Some major ions commonly found in water and soil such as: $Mg^{2+}$, $Ca^{2+}$, $Cl^-$, $Na^+$ were investigated for their effects on the assay performance with the following concentrations: NaCl up to 1000mM, $MgCl_2$ up to 50mM, $CaCl_2$ up to 50mM. The assay could tolerate $Mg^{2+}$, $Ca^{2+}$, $Cl^-$ and $Na^+$ at the tested concentrations. River samples collected from North Vietnam contain high levels of $Fe^{2+}$. The maximum concentration of $Fe^{2+}$ allowed for this assay was determined to be 1mM, above which reduction in color development and sensitivity was observed. Therefore, it was necessary to dilute these water samples in a buffer containing a chelating agent such as EDTA to reduce the effect of $Fe^{2+}$. Those obtained results were similar to the results of Lee *et al.* (*5*).

### *Effect of Sample Matrix*

Matrix effects occur due to the co-extraction of organic compounds from the samples, in addition to target analytes. In principle, a matrix effect can cause false positive or false negative results (*7*), but mainly false positives were found in the ELISA tests.

Many kinds of samples were examined to determine their effects on assay performance. Water samples could be analysed without further treatment. Soil, vegetables (cabbage, lettuce, bean, tomato, carrot and potato) and fruits (green grape, red grape, apple and pear) were found to be acceptable if their extracts were diluted 1 in 20 with distilled water (Figure 3). However, black tea extract affected the enzyme activity very strongly due to a high tannin content, even at a very high dilution. Among the absorbents tested, including alumina, silicagel, and polyvinylpyrrolidone (PVP), alumina was the most effective in removing interference from the black tea extract. Consequently, a protocol was optimized for tea extract to pass through an alumina column before an immunoassay. The matrix effect studies indicated that the reliable range of the kit for quantification of endosulfan would be 2–10 μg/L in the immunoassay.

## Recovery – Validation test

After the protocol for ELISA test had been set up to overcome matrix interference, the assay performed was validated by spike and recovery studies. The recovery of endosulfan by the cyclodiene ELISA was between 76-165% for the samples other than water (Figure 4). For water samples, a mean recovery of 95% by ELISA was obtained.

The inter-laboratory validation was performed between three analytical laboratories (SIAEP, CASE and UAF) for a cabbage and a grape sample spiked

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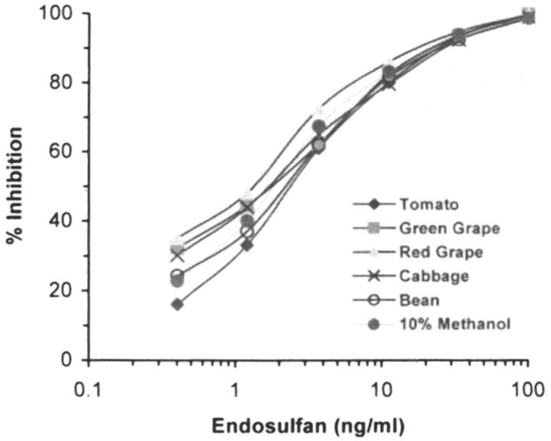

*Figure 3. Standard curves in sample matrix*

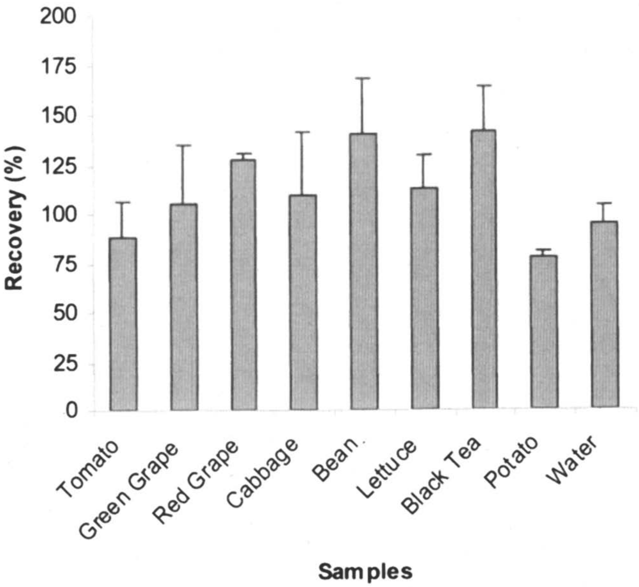

*Figure 4. Recovery of spiked endosulfan in samples*

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at different levels of endosulfan. The results shown in the Tables II and III indicated that the recovery of the ELISA kit was acceptable for screening purposes.

**Table II. Validation of ELISA kit for analysis of α-endosulfan spiked in cabbage**

| *Spiked cabbage (ppb)* | *Recovery (ppb) / (SD)* | | | | |
|---|---|---|---|---|---|
| | *CASE* | *SIAEP* | | *UAF* | |
| | *GC* | *GC* | *ELISA* | *GC* | *ELISA* |
| 222.4 | 223.05<br>(0.22) | 228.46<br>(2.6) | 253<br>(12.1) | 262.55<br>(4.2) | 227<br>(6.8) |
| 88.96 | 90.24<br>(0.51) | 95.39<br>(3.6) | 109<br>(9.2) | 100.22<br>(4.2) | 90<br>(7.8) |
| 133.44 | 134.03<br>(0.28) | 137.09<br>(2.2) | 165<br>(14.8) | 132.17<br>(4.1) | 122<br>(3.3) |

Triplicate samples were anlaysed in each analysis

SD: standard deviation

**Table III. Validation of ELISA kit for analysis of α-endosulfan spiked in grape**

| *Spiked grape (ppb)* | *Recovery (ppb) / (SD)* | | |
|---|---|---|---|
| | *CASE GC* | *UAF ELISA* | *SIAEP ELISA* |
| 107.2 | 108.4 (0.84) | 143 (4.3) | 153 (19.9) |
| 160.8 | 162.4 (0.50) | 221 (9.1) | 220 (9.1) |
| 268.0 | 267.3 (0.46) | 323 (4.3) | 340 (5.9) |

## Application of cyclodiene ELISA kit to monitor pesticide residues

More than 450 samples of soil, water, vegetables and fruits collected from North, South and Cental Vietnam were screened during 2002-2003 for cyclodiene contamination using the cyclodiene ELISA kit. The surveys and analysis by both ELISA and GC were conducted by the UAF and HUS. Approximately 10% of the positive samples by ELISA were independently re-validated by CASE using GC. The obtained results showed that almost all the positive samples (indicated by ELISA) were confirmed by GC to be positive and contaminated with endosulfan or/and endosulfan sulfate. As shown in Tables IV and V, the correlation between ELISA and GC was surprisingly good.

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**Table IV. Using ELISA kit to monitor cyclodiene contamination in water, soil, vegetable and fruit samples collected from the North, South and Centre of Vietnam (2002-2003)**

| *Sampling site* | *Sample* | *No. samples* | *ELISA results (analyzed by HUS and UAF)* | | |
|---|---|---|---|---|---|
| | | | *Positive samples (%)* | *Range of cyclodiene (µg/L)* | *Mean value (µg/L)* |
| Van Noi[1] | Water | 57 | 43 | 0.9 – 11.9 | 3.2 |
| | Soil | 50 | 78 | 8.0 - 300 | 178.0 |
| | Market vegetables | 16 | 25 | 16.3 - 380.3 | 159.2 |
| | Farm vegetables | 25 | 60 | 16.2 – 300 | 121.0 |
| Ninh Thuan[2] | Water | 20 | 90 | 0.4 -12 | 3.3 |
| | Soil | 22 | 100 | 7-3600 | 554.0 |
| | Farm grapes | 38 | 100 | 30.5 - 6000 | 771.0 |
| | Market grapes | 20 | 90 | 0.4 - 1283 | 254.0 |
| Hoc Mon[3] | Water | 50 | 68 | 0.4 -13.5 | 1.5 |
| | Soil | 51 | 43 | 0.4 - 61 | 8.0 |
| | Farm vegetables | 38 | 34 | 0.4 - 62 | 13.0 |
| | Market vegetables | 80 | 31 | 0.4 -109 | 5.0 |

[1]a vegetable cultivation area, close to Hanoi, Northern Vietnam

[2]a grape cultivation area, central Vietnam

[3]a vegetable cultivation area, close to Ho Chi Minh City, Southern Vietnam

Through the collaboration between SIAEP, HUS, UAF and CASE, the cyclodiene ELISA kit was successfully applied to monitoring of cyclodiene contamination in agri-product and environmental samples, assisting in risk assessment (*11*). For this survey, the application of the ELISA kit had reduced the cost of analysis with its high-throughput capacity for screening a large number of samples.

## Conclusion

Pesticide contamination in agricultural commodities and in the environment in Vietnam urgently needs strict regulation, because some farmers tend to abuse regulations for pesticide application. Endosulfan and other organochlorine

**Table V. GC results for positive samples collected from the North, South and Centre of Vietnam (2002-2003)**

| Sample | Code | *Cyclodiene residues (µg/L))* | |
|---|---|---|---|
| | | Analyzed by HUS/UAF/SIAEP ELISA | Analyzed by CASE GC/MS |
| Water | FW41 | 9.1 | 4.2 |
| | FW42 | 3.2 | 0.8 |
| | No 1 | 4.2 | 3.0 |
| | No 2 | 0.4 | 0.5 |
| | No 3 | 0.4 | 0.6 |
| Soil | FS36 | >240 | 323 |
| | FS46 | >240 | 555 |
| | FS51 | >240 | 357 |
| | D5 | 1360 | 1313 |
| | D7 | 552 | 456 |
| | D8 | 1600 | 1063 |
| | D9 | 880 | 555 |
| | D10 | 1048 | 1045 |
| | D16 | 928 | 919 |
| | D19 | 3600 | 2230 |
| Grape | N1 | 1640 | 1748 |
| | N11 | 1320 | 1544 |
| | N12 | 6000 | 5319 |
| | N13 | 3600 | 3111 |
| | N23 | 2600 | 2353 |
| | N30 | 560 | 565 |
| | N33 | 960 | 1028 |
| | N37 | 480 | 450 |

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pesticides are some of the pesticides commonly found in agricultural products. The ELISA-test developed by SIAEP-HCMC is able to detect endosulfan, its metabolites and other cyclodienes, and is hence suitable for screening purposes as employed elsewhere (*12-15*). The kits had been applied to screen hundreds of samples for their risk assessment. All the positive samples identified by ELISA were confirmed as positive by GC. The extensive validation confirmed that the ELISA kit can be applied for various sample types. Application of the ELISA test is highly effective, not only saving time and cost, but it is also simple to perform, requiring minimal technical skills to perform. Use of the ELISA has been successfully introduced to a large number of technical trainees in national workshops (*11*).

Due to its broad specificity, the ELISA test is only suitable for semi-quantitative screening purposes. Combining the high sensitivity and accuracy of gas chromatography with the high throughput ELISA, more accurate and precise results can be obtained cost-effectively and more quickly than GC analysis. The ELISA test has also been applied successfully for environmental water analysis in Ho Chi Minh City, confirming the cause of fish kills for the public record. It could also be applied widely to environmental monitoring for non-food crops such as cotton, similar to surveys conducted in other countries (*16*).

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## Chapter 16

# Monitoring Pesticides in the Paddy Field Ecosystem of North-Eastern Thailand for Environmental and Health Risks

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Contamination of the environment by pesticide application in paddy fields of NE Thailand was characterised by their toxicity to aquatic species, but specific classes of pesticides involved were not identified. Pesticides are dispersed in the environment and move through the food chain and may cause ecotoxicological and human health problems, hence the need for practical management tools. This research aimed at investigating a variety of techniques to screen contamination by pesticides in irrigation waters, and identify compounds that can be related to ecotoxicological effects in the paddy field ecosystem. It showed that pesticides such as desethylatrazine, atrazine, oxadiazon, dicofol, β-endosulfan and its degradation products (lactone and sulfate) are present at some sites. Pollution control authorities can develop monitoring tools for environmental management, to assist in providing sustainable agricultural practices in Thailand.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

## Introduction

Pesticide contamination associated with paddy field activities may pose significant environmental hazards for terrestrial and aquatic ecosystems. Effects may be observed via biomonitoring with both individual organisms and ecosystem function and structure. Pesticide monitoring is traditionally based on evaluations of individual pesticides identified through chemical analyses. In contrast, the ecotoxicity bioassay approach integrates the biological effects of all compounds present. Biomonitoring and ecotoxicological assessment of pesticides in the paddy field can provide a better indication of environmental effects than chemical analysis. An integrated approach is needed for pesticide management with paddy field ecosystems taking on board pesticide use and the concept of risk (*1*). The purpose of risk assessment is to measure the risks and guide the decision making process (*2*). It is necessary to distinguish relative risk, based on comparisons of different pesticides, and actual risk measured by the exposure, use of exposure data, modeling and field validation (*1*).

Many companies import and sell pesticides in Thailand. A factor that prevents transparent pesticide use and control is trade name proliferation (*3, 4*). The main pesticides used in paddy fields include monocrotophos (CAS:6923-22-4), metamidophos (CAS:10265-92-6), methyl parathion (CAS:298-00-0), methomyl (CAS:16752-77-5), glyphosate (CAS:1071-83-6), 2,4-D (CAS:94-75-7), atrazine (CAS:1912-24-9), ametryn (CAS:834-12-8) and paraquat (CAS:4685-14-7) (3). The highest risk from pesticide use to aquatic systems was associated with monoculture systems producing marketable crops (*5*).

The aquatic ecosystem is critical for evaluation of the effects of pesticides discharged from the paddy field ecosystem (*6*). Culture of fish in paddy fields is common; the species Nile tilapia (*Oreochromis niloticus*) is now found all over Thailand. Fish migrate into paddy fields during flooding, and are harvested when the rice matures. Multiple cropping of rice in much of the tropics exacerbates the problem of discharged irrigation waters. The impact of pesticides on the paddy field ecosystem can be assessed by monitoring commonly found species in the paddy field. A decrease in the population of a susceptible species due to the impact of a given pesticide can decrease species diversity and ultimately change the community structure (*7*).

## Analysis of Environmental Samples from Paddy Fields

Integrative sampling is required in order to detect the actual concentrations of pesticides in water. Some currently available approaches are as follows:

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1. Collection of a large volume of water, at least 1 L, followed by its concentration present by passage through a pre-filter to remove particulate matter and extraction with the Empore disc polar sampler (*8*) or by solid phase extraction columns; and
2. Deploying passive sampling or semipermeable membrane devices (SPMDs) at the sampling site held between 1 week and 1 month in the water column to concentrate the pesticides by integrative sampling (*9-11*). The SPMDs allow permeation of chemical species < 0.1 nm. A more recent version, developed at ENTOX, uses a specific polymeric material, polydimethylsiloxane (PDMS), with the property of permeability to concentrate pesticides in the water column. Concentrations of contaminants sequestered in SPMDs ($C_{SPMD}$) are converted to a water concentration ($C_W$) using a sampling rate ($R_S$ in L $d^{-1}$), determined in laboratory calibrations (*12*) using the following equation:

$$Cw = C_{SPMD} \times M_{SPMD}/Rs \times t$$

where $M_{SPMD}$ is the mass of the SPMD in grams and t is the sampling time in days. Rs values are available for a wide range of chemicals and can be corrected for water temperature. An internal calibration method compensates for site specific factors that affect sampler performance.

Instrumental analytical methods are required for pesticide analysis of prepared and extracted samples, prior to extensive clean-up procedures. Methods used are as follows: (i) Gas chromatography (GC) e.g. using electron capture detector (EC) for organochlorines; (ii) High performance liquid chromatography (HPLC); (iii) GC/MS; (iv) LC/MS-MS; and (v) Enzyme-linked immunosorbent assays (ELISA) (*1,13,14*). Methods can generally be obtained from the handbooks of the Association of Official Analytical Chemists (AOAC), the British Pharmacopoeia (BP) guidelines, the USEPA, ISO, AS and other Intergovernmental agencies such as from the OECD, UNEP and EU (*1*). Regardless of choice, all methods require validation of procedures within the local laboratory to demonstrate validity of quality assurance (QA/QC procedures), including use of verifiable standards.

The objective of the present study was to investigate the use of a variety of concentrating techniques to screen for pesticide residues in irrigation waters, identify important compounds that are relevant to ecotoxicological effects in the paddy field ecosystem, and to show their association with the presence of aquatic species diversity and type of farming activity. This work will provide the basis for development of an integrated risk assessment approach to improve management of pesticide application in N.E. Thailand paddy fields and indicate if any risks to environment and health exist.

## Material and Methods

### Sample Sites and Water Quality

Samples sites (Table I) were selected at the paddy field ecosystems in N.E. Thailand within 50 km from the city of Khon Kaen, 450 km NE from the capital Bangkok. All sampling sites, excepting Site 1, which used groundwater and no synthetic pesticides (botanical pesticide such as Neem extract- *Azadiracta siamesis* is used), received irrigation waters that were subsequently discharged into tributaries of the Mekong River such as the Mae Nam Phong. This sub-region is characterized by the presence of high clay floodplain soils with broad lateritic features giving an overall low organic carbon in sediment (<2%) (*15*). The catchment area of the Mekong River within Thailand is 188,623 km$^{2.}$ The annual rainfall at Khon Kaen meteorological station (Lat 16°26′00 Long 102°50′00) is 1,113 mm and average temperature is 27.5 °C. Average rainfall for May-September is 1000 mm corresponding to the monsoonal period and may exceed 200 mm/month. The dry season is from October to April.

**Table I. Sample sites nearby Khon Kaen, N.E. Thailand**

| *Sample site* | *Location (latitude and longitude)* | *Comments* |
|---|---|---|
| Site 1<br>Organic farming pond | Nondongton Village, Amphur Nampong (16° 47′ 23 N, 102° 46′ 01 E) | Groundwater and natural pesticide (Neem) use. Low turbidity. |
| Site 2<br>Conventional paddy farming | Nondongton Village, Amphur Nampong (16° 47′ 15 N, 102° 46′ 05 E) | Adjacent to Site 1 Range of pesticides used. Turbid water. |
| Site 3<br>Paddy, heavy pesticide use | Beung Chim Amphur Muang (16° 26′ 39 N, 102° 56′ 04 E) | Adjacent to main irrigation canal. Turbid water. |
| Site 4<br>Orchard - heavy pesticide use | Beung Chim Amphur Muang (16° 26′ 45 N,102° 55′ 59 E) | Nearby (250m) Site 3 and irrigation channel. Very turbid water. |
| Site 5<br>Irrigation Canal for major paddy field area | Ban Dornyang (16° 27′ 49 N,102° 52′ 32 E) | Main channel to large paddy field area. Low turbidity. |

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## Collection of Water Samples, Sediments and Passive Samplers Deployment

Samples were collected between July-December 2005, near the end of the wet season. Field blanks were taken at sampling sites and treated in the same way as other samples to monitor any contamination during sampling. Field spikes were not used as pesticides present were not yet identified. Sampling procedures were those used routinely by ENTOX/Queensland Health Scientific Services (QHSS) pesticide analytical laboratory, Coopers Plains, Brisbane, Australia. All methods were validated in the laboratory for pesticide recovery, accuracy and losses. HPLC grade acetone and methanol were obtained from a local supplier in Khon Kaen. Milli Q grade water was passed through a prepared Empore disc to remove any organic contamination and also used as treatment blanks.

Water samples were collected on 31 July 2005 in prepared 2.5 L glass bottles. and kept on ice in insulated containers until returned to the laboratory refrigerator at the Khon Kaen University laboratory. Water samples were filtered through Whatman GF/A glass microfibre filters prior to concentrating of pesticide residues on prepared Empore disks. A number of water samples clogged the filter paper and were changed. The volume filtered was 1000 mL.

The Empore disks (Varian/Phenomenex) SDB-RPS were prepared initially by soaking in 20 mL HPLC grade methanol/ disk for 30 min. The contents of the sample bottle plus three consecutive rinses were passed through the Empore disc. The volume of sample varied from 100-1000 mL and 2500 mL for blanks. Commercially available Oasis Sample Extraction Cartridges (Waters HLB 12cc 500 mg LP Extraction Cartridges) were also used to extract 500 mL of sample.

Sediment samples were collected at each site on 13 November 2005. Representative samples comprised 5 portions taken 0-5 cm below the water interface material. Each sediment sample was kept refrigerated until dispatched for analysis.

The PDMS passive samplers (strips 2.5 cm X 92 cm and thickness 350 μm) have been demonstrated at ENTOX to give quantitative recovery of all pesticides from the water column except for phenoxy esters. Each PDMS was pre-extracted in 900 mL of hexane for 3 x 24 h periods at the ENTOX/QHSS laboratory. The PDMS were dried under high purity nitrogen gas steam, then wrapped in acetone rinsed aluminium foil immediately after fabrication and stored at –17 $^0$C until dispatched to Khon Kaen and deployed on 13 November 2005. Each strip of PDMS was wrapped around two steel rods and placed underwater at each site below a depth of 0.25 m and left in placed for 2 weeks, then removed on 27 November 2005. Each strip of PDMS was placed immediately in pre-cleaned acetone rinsed Al foil envelopes and kept in the refrigerator until dispatched for analysis.

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Following collection, preparation and storage, items for analysis of pesticides were sent together with appropriate AQIS permit details for quarantine clearance from Khon Kaen to the ENTOX/QHSS pesticide analytical laboratory of QHSS Brisbane, QLD Australia for sample processing and analysis in the NATA accredited laboratory (according to ISO 17025). QHSS Internal Method 16313 based on USEPA Method EPA 503/6-90-004 was used for determination of pesticides in sediment. Organic carbon was also measured in sediments by QHSS using a LECO C200 carbon analyser.

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### Biomonitoring of the Paddy Field Ecosystem

Biomonitoring was conducted on aquatic insects, zooplankton and phytoplankton in rice paddy water and sediment from the sample sites. Aquatic biota comprising aquatic insects, phytoplankton and zooplankton were also collected each month and at each site during the rice production season (May-September 2005). The collections were sorted and numbers of different species counted. Using the aquatic biota data from each site, the Shannon-Wiener diversity index (*16*) was calculated in order to relate the diversity of the sites with their degree of pollution by pesticides.

$$\text{Shannon-Wiener diversity index } (H') = -\Sigma\, Pi \log Pi$$

where $Pi = ni / N$ where $P_i$ is proportion of individuals of $i^{th}$ species
i is the species concerned
n = number of individuals for each species
N = total number of individuals found

## Results

### Water Quality of Sampling Sites

Table II gives the water quality parameters measured by field instruments at each sampling site (13 November 2005), calibrated according to manufacturer's instructions. There was visual evidence of high turbidity and colloidal/suspended matter in most water samples, excepting at Site 5. Ambient water temperature was typically high at all sites but dissolved oxygen concentrations indicated were near saturated conditions. Site 1 had more elevated EC compared with lower EC for irrigation waters (Sites 2, 3 & 5) and slightly acidic pH, inferring an effect from the soil due, perhaps to groundwater and organic farming techniques.

Table. II Water quality parameters for each sampling site

| *Location* | *Time (hr)* | *Temperature (°C)* | *Dissolved Oxygen (mg/L)* | *pH* | *EC (μS/cm)* |
|---|---|---|---|---|---|
| Site 1 | 12:30 | 30.4 | 5.25 | 5.4 | 2160 |
| Site 2 | 13:20 | 32.2 | 6.30 | 6.1 | 489 |
| Site 3 | 16:06 | 35.3 | 4.8 | 6.9 | 174 |
| Site 4 | 16:30 | 34.0 | 6.0 | 7.3 | 1000 |
| Site 5 | 17:23 | 32.5 | 6.2 | 7.35 | 224 |

The higher EC found at Site 4 is not explained by variation in water source as pH at both Sites 4 and 5 (near each other) were similar, but were using the same irrigation water source. The higher EC at Site 4 resulted from the addition of soluble salts to irrigation water. All sites receiving irrigation water had relatively high turbidity, excepting at Site 1 and the inflowing paddy irrigation water at Site 5 (Table II). The presence of high turbidity irrigation waters containing fine clay particles from local floodplain soils with low EC appear to be indicative of used paddy water. These clay materials are important with respect to their capacity to absorb pesticides when coated with organic carbon.

## Pesticides in Water Samples

The results for detected pesticides in water samples are summarized in Table III. Results for all other samples and blanks were at or below detection limits.

Table III. Concentrations of pesticides (ng/L) in water samples and treatments with pre-filter (GF/A), Empore disc and SPE (Oasis) columns.

| *Pesticide* | *Site 3 Pre-filter GF/A* | *Site 3 (Empore disc)* | *Site 3 (SPE column)* | *Site 5 (SPE column)* | *All Blanks* |
|---|---|---|---|---|---|
| desethylatrazine | <10 | <20 | 37 | 48 | <10 |
| atrazine | <10 | <20 | 91 | <20 | <10 |
| endosulfan lactone | <10 | <20 | 96 | <20 | <10 |
| oxadiazon | <10 | <20 | 106 | <20 | <10 |
| endosulfan beta | <10 | 70 | 519 | <20 | <10 |
| endosulfan sulfate | 21 | 26 | 870 | <20 | <10 |

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Both the pre-filter and SPE column results include particulate matter whereas the Empore disc results are only for pesticides in solution. The SPE columns will also trap clay particles. The results show that pesticides were only detected at Sites 3 and 5. At Site 3 the pesticides were limited to β-endosulfan (CAS:33213-65-9) and endosulfan sulfate (CAS:1031-07-8) and appeared to be bound to suspended particles; the presence of suspended forms is assumed from the comparison of lower pre-filter concentration(s) present as particulate form (>0.2 μm) when compared with the SPE column results and the limited detection of soluble forms. The isolated presence of desethylatrazine (CAS:6190-65-4) in the SPE column only at Site 5 also implies an association with suspended particles. Thus grab water samples and the specific treatments applied enabled the identification of recent pesticide application.

## Pesticides and Organic Carbon in Sediment Samples

The results for pesticide and % organic carbon concentrations in sediment samples are summarized in Table IV. Paddy fields (Sites 2 and 3) accumulate organic carbon while other sites have typical concentrations of organic carbon. Only endosulfan sulfate was detected at Site 3. All other pesticides were below their respective detection limits: organophosphorus pesticides <5 μg/kg; synthetic pyrethroids <20 μg/kg; organochlorine pesticides <5 μg/kg; atrazine, diuron (CAS:330-54-1), flumeturon (CAS:2164-17-2), hexazinone (CAS:51235-04-2), tebuthiuron (CAS:34014-18-1), ametryn and prometryn (CAS:7287-19-6) all <1 μg/kg.

**Table IV. Pesticides and organic carbon in sediment**

| *Sample Number* | *Site 1* | *Site 2* | *Site 3* | *Site 4* | *Site 5* |
|---|---|---|---|---|---|
| Organic C (%)) | 0.67 | 1.1 | 2.4 | 0.37 | 0.21 |
| endosulfan sulfate (μg/kg) | <5 | <5 | 9 | <5 | <5 |

These results provide an important comparison with the water results (Table V) as they show that the sediment is not acting as a sink, excepting for endosulfan sulfate at Site 3. If other pesticides were present in sediment, they did not have a long residence time. The $t_{1/2}$ sediment for endosulfan is quoted as 70 d and for water 4 d (*1,17*). This implies that the results in Table IV are indicating relatively recent use of endosulfan and those other pesticides, if used, have broken down quickly.

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**Table V. Pesticides in water (total ng), found in PDMS after 2 weeks deployment**

| *Sample Reference* | *PDMS Blank 1* | *PDMS Blank 2* | *Laboratory Blank* | *Site 1* | *Site2* | *Site 4* |
|---|---|---|---|---|---|---|
| Analyte / Units | Total ng's | Total ng's | Total ng's | Total ng's | Total ng's | Total ng's |
| p,p'-dicofol breakdown | <30 | <30 | <30 | <30 | 100 | <30 |
| endosulfan lactone | <20 | <20 | <20 | <20 | 20 | <20 |
| oxadiazon | <10 | <10 | <10 | <10 | <10 | 110 |
| endosulfan sulfate | <30 | <30 | <30 | <30 | 420 | 200 |

## Pesticide in Passive Samplers

PDMS appears to be a potentially useful passive sampler material which can be improved by extending the sampling period to 30 d. Some pesticides were detected in the PDMS after 2 weeks deployment. At Site 2, an interesting finding was the breakdown product of dicofol (Table V). Dicofol (CAS:115-32-2) has 2 isomers: p,p'-dicofol (80%) and o,p'-dicofol (20%). The p,p'-dicofol breakdown product was quantified as a standard was available but, initially o,p'-dicofol could not be quantified by GC-MS as no standard was available (due to exclusion of OC pesticides from Australia). It is therefore possible that the concentration for dicofol were underestimated due to unknown amounts of o,p'-dicofol. Its amount may be 20% more than shown. Dicofol and oxadiazon (CAS:19666-30-9) concentrations were subsequently estimated from dacthal (CAS:1861-32-1) and p,p'-methoxychlor (CAS:72-43-5), respectively, due to their proximity in Log Kow values.

Dicofol is an organochlorine miticide, structurally related to DDT but with little insecticidal activity (*18*). It is highly toxic to aquatic life and can cause egg-shell thinning in some bird species but it remains in use in many developing countries especially against organophosphorus-resistant mites, although it has been voluntarily withdrawn in developed countries. As dicofol $t_{1/2}$ in the environment is reported to be 60 d (*18*), its presence at Site 2 indicates local and relatively recent use. Oxadiazon (*19*) is a commonly used herbicide in Asia and Thailand (*20*).

The concentrations of pesticides in water for the PDMS data (Table VI) were estimated by using the sampling rate for SPMDs at 26 $^{0}$C as described (*12*). Note there is some approximation with ambient temperature at the sites

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measured at 30-35°C (Table IV). The sampling rate for endosulfan sulfate ($R_s$) was considered to be between 1-5 L/day, as it is still in a linear uptake phase after 2 weeks of PDMS deployment ( ie the concentration in SPMD over time).

The calculated concentrations (ng/L) are presented in Table VI as an interval for the upper and lower sampling rates. The amount of endosulfan lactone was close to the detection limit and could not be further determined as the sampling rate for the estimation of its concentration in water is not yet available. Nevertheless the usefulness of the passive sampler approach is demonstrated as the levels measured are a better estimate of aquatic biota exposure concentrations.

**Table VI. Pesticides concentrations in water (ng/L) estimated from Table V**

| *Sample Reference* | *PDMS Blank 1* | *PDMS Blank 2* | *Laboratory Blank* | *Site 1* | *Site2* | *Site 4* |
|---|---|---|---|---|---|---|
| Analyte / Units | ng/L | ng/L | ng/L | ng/L | ng/L | ng/L |
| p,p'-dicofol breakdown | <1 | <1 | <1 | <1 | 4 | <1 |
| endosulfan lactone | <1 | <1 | <1 | <1 | <1 | <1 |
| oxadiazon | <0.3 | <0.3 | <0.3 | <0.3 | <0.3 | 2.8 |
| endosulfan sulfate | <1 | <1 | <1 | <1 | 20-30* | 10-14* |

Comparison of the PDMS results for Sites 1, 2 and 4 with those for water (Table III) and sediment (Table IV) shows that Site 1 had no detectable pesticides on PDMS, thus confirming that conventional pesticides were not used at this site. At Site 2, the presence of various pesticides including the organochlorine dicofol and endosulfan were detected by PDMS but not in sediment or the water sample. This shows the usefulness of PDMS in detecting what is actually present. Pesticides detected at Site 4 were similar to those found in the water sample from the nearby Site 3. It was unfortunate that the PDMS at Site 3 was lost as it is clear that the PDMS would have detected pesticides present in solution including β-endosulfan and its degradation products, oxadiazon, atrazine and desethylatrazine, all of which were detected in the water sample at relatively high concentrations. Following a visit to Site 3 on 9 December 2005, it was noted that the water level in the irrigation channel had fallen by about 3 m due to a flow controller being opened at the bottom and dropping the water to a level that was not previously visible. It is likely that the passive sampler was simply swept away. The PDMS results also indicate their potential application to measure levels of exposure that aquatic biota would experience. The findings indicate the need to better locate the passive samplers to prevent loss. The results for passive sampling are the first results of their kind from N.E. Thailand.

## Ecological Effects

Ecotoxicity of pesticides used in paddy fields to non-target organisms was further examined by assessing some ecotoxicological responses to Nile tilapia (*Oreochromis niloticus*), a commonly found fish that is taken as a food source, and to a waterflea *(Moina macrocapa* Straus). Common zooplankton found in the aquatic ecosystem are also food for fish. The toxicity of various pesticides used in the paddy field, β-endosulfan, chlorpyrifos (CAS:2921-88-2), metamidophos, carbosulfan (CAS:55285-14-8), were studied by static bioassays under laboratory conditions at Khon Kaen University. The observed mortality data of the acute tests was used to calculate median lethal concentration (48-h $LC_{50}$) with 95 % confidential limits. The results showed that 48-h $LC_{50}$ of β-endosulfan, chlorpyrifos, metamidophos and carbosulfan for Nile tilapia were $5.8 \times 10^{-4}$, $2.4 \times 10^{-2}$, 3.2 and 0.35 mg/L, respectively. The results also showed that 48-h $LC_{50}$ of malathion (CAS:121-75-5) and chlorpyrifos for water flea were 10.4 (9.1-11.9 c.i.) and 0.08 (0.03-0.2 c.i.) μg/L, respectively.

This study suggests that ecotoxicological assessment of pesticides in the paddy field ecosystem can provide further understanding of their effect on aquatic biota and can provide a basis for better management of their environmental impact. In the absence of local ecotoxicity data, other published data can be used to make first tier comparisons with concentration data. The usefulness of this approach can be demonstrated by comparing published $LC_{50}$ data (Table VII) with the measured concentration of detected pesticides (Tables IV-VI), all of which lie below $LC_{50}$ levels. However, the effects of lower levels of pesticide residues in paddy water should be considered as the results from this study on the sublethal effects of malathion and chlorpyrifos on water flea by renewable bioassay method showed that malathion at concentration 0.5 μg/L and chlorpyrifos at concentrations 0.00025–0.00045 μg/L had effects on the reproduction of water flea.

Table VIII shows the Shannon-Wiener diversity (Biodiversity Indices) for aquatic insects at two paddy fields. A gradual increase in aquatic insect biodiversity is apparent, peaking at the end of the wet season by the enhanced biomass and availability of water and nutrients. Apart from pesticides used in the paddy field at Site 3, such ecological impacts may result from the combined effects of other pollutants and environmental factors.

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**Table VII. Summary of published $LC_{50}$ data for detected pesticides.**

| *Pesticide* | *Species* | *$LC_{50}$ (mg/L)* |
|---|---|---|
| dicofol (*18*) | Mysid shrimp | 0.14 (96 h) |
| | Fathead minnow | 0.183 (96 h) |
| | Channel catfish | 0.30 (96 h) |
| | Bluegill | 0.51 (96 h) |
| | Eastern oyster embryo | 15.1 (96 h) |
| oxadiazon (*19*) | Rainbow trout | 1.2 (96 h) |
| | Algae | 6-3000 ($EC_{50}$ 96 h) |
| endosulfan (*1,17*) | Frogs | 2-12 (acute) |
| | Fish | 0.3-5085 (acute) |
| | Crustaceans | 7-7000 (acute) |

**Table VIII. Biodiversity Index (*H'*) for aquatic insects in paddy fields**

| *Site type* | *May* | *June* | *July* | *August* | *September* | *October* |
|---|---|---|---|---|---|---|
| Organic farming pond – Site 1 | 0 | 0 | 4.14 | 4.16 | 4.07 | 5.43 |
| Paddy, heavy pesticide use – Site 3 | 0 | 0 | 3.90 | 3.19 | 3.13 | 5.76 |

The results in Table IX give a summary of the Shannon-Weiner Biodiversity Indices for phytoplankton and zooplankton species found in water at each of Sites 1-4 in October 2005. No collection was made at Site 5 as the irrigation channel water was fast flowing. The Biodiversity Index declined from Site 1 (highest diversity-no conventional pesticide use) through to Site 4 and significantly including Site 3. There is a clear association between decreasing biodiversity indices and increasing pesticide use as demonstrated by the pesticide data for water, sediment and PDMS (Tables III-VI).

## Discussion

### Sampling Approach to Detect Pesticide Use

The use of grab water samples and the specific treatments applied enable the identification of recent pesticide application based on their relatively high concentrations. The persistence of pesticides is most easily identified from sediment samples. The comparison of results from active sampling, sediment analysis and PDMS analysis shows that passive sampling is the best technique for monitoring pesticides in the future. The results indicated that pesticides were not building up in sediment except for endosulfan sulfate (Table III and IV) at the more contaminated paddy field site. The results also show the concentrations that organisms are being exposed to. Endosulfan stands out clearly as a pesticide of high priority for risk assessment framework development, paralleling the situation in Vietnam (*1*).

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**Table IX. Summary of Biodiversity Indices (*H'*) for phytoplankton and zooplankton at Sites 1-4.**

| *Location* | *Farming characteristics* | *Phytoplankton* | Zooplankton |
|---|---|---|---|
| Site 1 | Organic farming pond<br>No synthetic pesticides - only botanical pesticides (Neem) | 7.18 | 9.39 |
| Site 2 | Conventional paddy field<br>Pesticides used include: carbofuran, endosulfan, chlorpyrifos, dicofol, malathion, monocrotophos, synthetic pyrethroids | 5.76 | 8.26 |
| Site 3 | Paddy<br>Heavy pesticide usage: endosulfan and oxadiazon | 4.18 | 5.31 |
| Site 4 | Orchard<br>Heavy pesticide usage: endosulfan and oxadiazon | 1.43 | 2.77 |

## Biomonitoring and Ecotoxicity

Integrating water concentration data from PDMS or grab sample with biodiversity index and ecotoxicity data for local aquatic species at the site specific level is a key goal. The usefulness of biodiversity index data to demonstrate pesticide effect was clearly demonstrated by comparing Site 1 with all other sites using pesticides. It is also clear that pesticide use has an effect on the abundance and diversity of aquatic species in paddy field ecosystems. Ecotoxicity assessment is needed to identify which local species are most sensitive to exposure and could be suitable for testing purposes. The value of using a biological tool, a simple tool such as biodiversity and ecotoxicity data, is to monitor ecological effects of pollutants (pesticides or other) in paddies and other agricultural landuses. It would be suitable to use biological monitoring for assessing risks from pesticide use in developing countries, apart from Thailand, or elsewhere in the Mekong Basin, which cannot afford expensive chemical residue analysis on a routine basis. Biological monitoring can detect changes in the ecology of particular areas at very low cost (*7*).

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## Conclusions

There is a need for biomonitoring and ecotoxicological assessment of pesticide use in paddy fields together with innovative sampling approaches such as using passive sampling devices. The results from this study have provided useful information for the pollution control authorities to develop a tool for environmental management and to assist in providing sustainable agricultural practices in Thailand.

## Acknowledgements

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## Chapter 17

# Simultaneous Measurement of *Bacillus thuringiensis* Cry1Ab and Cry3B Proteins in Corn Extracts

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Transgenic corn hybrids have been developed which contain synthetic genes for the production of *Bacillus thuringiensis* insecticidal proteins. Two of these proteins are Cry1Ab and Cry3B. Enzyme-linked immunosorbent assays (ELISA) and immunochromatographic lateral flow assays, which are sensitive, specific and easy to perform, have been developed for Cry1Ab and Cry3B proteins in corn extracts. In the present work, we describe a sandwich fluorescent covalent microsphere immunoassay (FCMIA) to simultaneously measure Cry1Ab and Cry3B proteins in corn extracts. Mixtures of genetically modified organism (GMO) and non-GMO corn were prepared and extracted. The extracts were diluted and Cry1Ab and Cry3B were measured by FCMIA. Measurement of pure Cry1Ab protein by FCMIA gave a minimum detectable concentration of 0.1 ng/mL. There were no significant differences between the assays when performed singly vs. multiplexed ($P>0.588$). When the observed dilutions from the four parameter logistic fits were compared to the known dilutions, highly linear relationships were observed ($r = 0.984$ and $0.983$, $P<0.001$) over a dilution range

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

of 1 to 100. These data give proof of principle that FCMIA can be used to simultaneously measure multiple GMO proteins in corn and may be a valuable adjunct to existing assays when the number of GMO pesticidal proteins to be measured becomes large.

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# Introduction

*Bacillus thuringiensis* (Bt, named for the Thuringia region of Germany) is a naturally occurring bacterial organism that has been known to man since the 19th century (*1*). Bt is a spore-forming bacteria (related to *Bacillus anthracis)* that, during spore formation, produces a crystalline structure. Within these crystalline inclusions are pesticidal toxins (cry(stal); Cry) named Cry proteins which were numbered in the order in which they were discovered. Genes encoding these δ-endotoxins have been transferred to major crops (such as corn, rice, cotton, potato, tomato, tobacco, soybean, etc.) which then produce Bt toxins (*2*). When ingested by insects, these toxins attack the midgut (*2*), resulting in the interruption of feeding by the insect, as well as gut paralysis, destruction of midgut epithelium and disruption of cell membrane permeability (*2*).

Plants which have been genetically modified with Bt genes are regulated in the United States by three agencies: U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), the U.S. Environmental Protection Agency (EPA), and the Department of Health and Human Services' Food and Drug Administration (FDA). For example, USDA-APHIS under the Plant Protection Act (PPA, 7 USC 7701-7772) regulates corn as a "regulated article", based on the procedures used to introduce or express the Bt gene. EPA is responsible for setting the amounts or levels of pesticide residue that may safely be in food or feed. Developers of Bt crops also consult with FDA about possible other, unintended, changes to the food or feed, for example possible changes in nutritional composition or levels of native toxicants. Although this consultation is voluntary, all of the food/feed products commercialized to date have gone through the consultation process (*3*). Therefore efficient methods are needed to estimate the quantities of these toxic proteins.

In the present work, we evaluated the simultaneous measurement of Cry1Ab (e.g., Mon810 and Bt11 - Yieldgard™, Monsanto, St. Louis, MO) and Cry3B (e.g., MON863 YieldGard™ Rootworm) in corn extracts using a newly developed fluorescent covalent microsphere immunoassay (FCMIA) (*4, 5, 6, 7, 8*).

## Methods and Materials

### Antibodies and Pure Protein

Polyclonal rabbit anti-Cry1Ab, 2.7 mg/mL, polyclonal goat anti-Cry1Ab, 2.7 mg/mL, polyclonal rabbit anti-Cry3B, 5.7 mg/mL and mouse monoclonal anti-Cry3B, 5.1 mg/mL, purified Cry1Ab, as well as ground genetically modified organisms (GMO) (Cry1Ab and Cry 3B) and non-GMO corn were obtained from EnviroLogix (Portland, ME). All antibody solutions were dialyzed (Slide-A-Lyser 30 kD cassette, [Pierce Biotechnology, Rockford, IL] at 4°C against 50% diluted PBS [PBS pH 7.4, Sigma Chemical Co., St. Louis, MO]) to remove the sodium azide preservative in the commercial preparations.

### Preparation of Capture Microspheres

The polyclonal rabbit anti-Cry1Ab and mouse monoclonal anti-Cry3B were used as capture antibodies. They were conjugated to two sets of microspheres ($2.5 \times 10^6$, Luminex Corp., Austin, TX). The microspheres were 5.6 μm in diameter and composed of polystyrene, divinyl benzene and methacrylic acid, which provided surface carboxylate functionality for covalent attachment of biomolecules. Internally, the microspheres were dyed with red and infrared emitting fluorochromes. By proportioning the concentrations of each fluorochrome, spectrally addressable microsphere sets were obtained. Prior to conjugation, the microspheres were activated. Briefly, the microspheres were pelleted (5,000 x g for 2 min) in 1.5 mL centrifuge tubes using a microcentrifuge (Eppendorf, Hamburg, Germany). They were then resuspended by sonication [mini sonicator, Cole Parmer, Vernon Hills, IL], and gentle vortexing [VWR, Intl., West Chester, PA]) in 80 μL activation buffer (0.1M $NaH_2PO_4$, pH 6.2) to which 10 μL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Pierce Chemical Company, Rockford, IL), and 10 μL of 50 mg/mL N-hydroxysulfosuccinimide, sodium salt (sulfo-NHS, Pierce Chemical Company) were added. The mixture was allowed to incubate for 20 minutes at room temperature. The microspheres were then washed twice in 500 μL coupling buffer (0.05 M 2-[N-morpholino]ethanesulfonic acid, MES, [Sigma Chemical Co.], pH 5.0) and solutions of anti-Cry1Ab, or anti-Cry3B (50 μg/mL) in 500 μl coupling buffer was added and incubated for 2 hrs at room temperature. The coupled microspheres were then washed twice in 1 mL wash buffer (PBS containing 0 .05 % Tween 20 [Sigma #3563] ) and stored in 0.5 mL PBS-BSA (PBS, 1% BSA [Sigma #3688], 0.05% Na Azide [EM Sciences, Cherry Hill, NJ], pH 7.4). Microsphere concentrations were determined using a hemacytometer (Bright Line, VWR, Intl.).

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## Biotinylation of Detector Antibodies

Goat anti-Cry1Ab and rabbit anti-Cry3B were biotinylated using a commercially available biotinylation kit (BiotinTag Micro Biotinylation Kit, Sigma Chemical Co., St. Louis, MO) following the manufacturer's directions.

## GMO and Non-GMO Corn Mixtures and Extraction

Mixtures of GMO and non-GMO corn were prepared to yield 10% and 50% by dry weight two component mixtures (e.g. 10% Cry 1Ab corn, 90% non-GMO corn). In addition, 50% Cry1Ab and 50% Cry3B mixtures were made. Three component mixtures of corn were also prepared (e.g., 10% Cry1Ab corn +10% Cry3B corn +80% non-GMO corn). Two grams of each of these mixtures, as well as 100% Cry1Ab and 100% Cry3B, were extracted in 5 mL extraction buffer (PBS-0.55% Tween 20). The mixture was then vortexed until all solids were suspended and then left undisturbed for 1 hour at room temperature. The mixture was re-vortexed and the solids allowed to settle until the extraction liquid became visibly clear. An aliquot of the extraction liquid was pressure filtered (0.45 μ Acrodisc) and placed in a clean vial. The primary corn extracts were further diluted (PBS-BSA) 1:3 and 1:10. This effectively yielded 1, 3.3, 5, 10, 16.7 and 50% mixtures which were extracted from a mixture of GMO corn and either Cry1Ab or Cry3B corn or non-GMO corn and Cry1Ab and Cry3B corn. Blanks were prepared by diluting extraction buffer in the same manner as the corn extracts.

## Monoplex and Diplex Suspension Array Measurements

The Bio-Plex suspension array used to measure fluorescence (Bio-Rad Laboratories, Hercules, CA) was systematically calibrated and validated before each run using a maintenance, calibration, and validation plate, and standards supplied by the manufacturer. Conjugated microspheres (50 μL), at a working concentration of $1x10^5$ microspheres/mL for each microsphere type in PBS-BSA, were added to the wells of a 1.2 μm filter membrane microtiter plate (Millipore Corp., Part #MABVN1250, Bedford, MA) and the liquid aspirated by use of a vacuum manifold filtration system (Millipore, Part #MAVM09601). For each experiment, 50 μL (in duplicate) of pure Cry1Ab protein, corn extract, diluted extract or control buffer were added to either one (monoplex) or a mixture (diplex) of microspheres in the wells of the filter membrane microtiter plate and incubated for 30 min at 37° C with shaking. The contents of the wells were aspirated and the wells were washed three times with 200 μL wash buffer.

Fifty µL of the biotinylated detector antibody mixture in PBS/BSA was added to the wells and incubated for 30 min at 37° C. The contents of the wells were aspirated and the wells were washed three times with 200 µL wash buffer. An aliquot (50 µL) of a 4 µg/mL solution of streptavidin R-phycoerythrin (PE, Molecular Probes, Eugene, OR) in dilution buffer (PBS/BSA) was added to the wells of the plate and incubated for 15 min at 37° C. The wells were again washed three times with 200 µL wash buffer and resuspended in 100 µL wash buffer. The plate was shaken vigorously for approximately one min to disperse the microspheres and was placed into the autosampler platform of the BioRad Bioplex Suspension Array instrument using software, calibration microspheres, and sheath fluid supplied by the manufacturer. The instrument was programmed to collect data from 100 microspheres (classified by their internal fluorescence ratio) and acquire the median fluorescence intensity (MFI) of the microsphere-antibody-toxin-antibody-biotin-R-PE complex(es). The MFI response to Cry1Ab protein at 0, 0.1, 0.3, 1, 3, 10, 30 and 100 ng/mL was also evaluated in a sandwich FCMIA.

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## Data Analyses

For Cry1Ab, a standard curve was constructed from 4-parameter logistic log fits (4-PL) of MFI vs. the logarithm of ng/mL (*5, 7, 8*) (SigmaPlot, Systat, Point Richmond, CA) using the following relationship:

$$Y = \{(A - D) / [1 + (X/C^{B})]\}$$

where A is the maximum MFI response, B is the slope at the inflection point, C the concentration of analyte at 50% maximal MFI, and D is maximal MFI. The Cry1Ab minimum detectable concentration (MDC) was calculated from the intersection of the asymptote of the regression's 95% confidence interval (CI) with the regression line (*6, 8, 9*). Assessment of the "goodness of fit" and the dynamic ranges of the assays were investigated by evaluating the fit of the standards data to the 4-PL model by "standards recovery", calculated by evaluating interpolated results from each 4-PL fit and comparing it to the actual concentration of Cry1Ab added to the system (*10*) using the following relationship:

$$\text{Percent recovery} = 100 \text{ x} \left( \frac{\text{Observed Concentration from 4PL fit}}{\text{Expected Concentration from 4PL fit}} \right)$$

The resultant data were analyzed for linearity by regression analysis (*7*). Extracts from GMO corn:non-GMO corn two and three component mixtures were either analyzed or diluted and analyzed as monoplex or diplex FCMIAs (1.0, 3.3, 5.0, 10, 16.7 and 50% GMO:non-GMO mixtures) and linear regressions of percentage GMO corn:non-GMO corn vs. MFI evaluated. Differences in MFI when the assays were run as monoplex or duplex were investigated using one-way repeated measures analysis of variance (SPSS, Chicago, IL).

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## Results

The FCMIA for Cry1Ab had an excellent fit to the 4-PL model (Figure 1A, r=0.998, P <0.0001). When the observed vs. expected concentration returned from the 4-PL model was evaluated, highly linear responses (r=0.996, P<0.001) were observed (Figure 1B). The MDC for Cry1Ab was 0.1 ng/mL. When extracts and dilutions from increasing ratios of Cry1Ab corn:non-GMO corn were evaluated in a monoplex format FCMIA (Figure 2 A), a linear relationship (MFI = [3217.7*Log(percent Cry1Ab) - 100.33]) was observed (r=0.983, P<0.001). Similarly, extracts and dilutions from increasing ratios of Cry3B corn:non-GMO corn were evaluated (Figure 2 B) in a monoplex format FCMIA, a linear relationship (MFI = [3221.2*Log(percent Cry3B)+7470.4]) was observed (r=0.984, P<0.001). When extracts and extract dilutions of mixtures of Cry1Ab corn, Cry3B corn, and non-GMO corn were evaluated (Figure 3) in a diplex FCMIA, the resulting linear relationships [(MFI = [3007.3*Log(percent Cry1Ab) + 173.6]), ( r=0.965, P<0.002) and (MFI = [4177.4*Log(percent Cry3B) + 6807.3]), (r=.940, P=0.005)] were observed. MFI responses for the FCMIA assays executed in monoplex vs. diplex modes were are shown in Figures 4 A and B. One way repeated measures ANOVAs of these data demonstrate that no statistically significant differences were observed related to performance of diplex vs. monoplex FCMIA for either Cry3B non-GMO corn or Cry1Ab non-GMO corn mixtures (P=0.602 and 0.588, respectively). These results strongly suggest that there is no cross-reactivity between Cry1Ab and Cry3B.

## Discussion

Genetic modifications are made to plants generally for two reasons, either to impart pesticidal properties to the plant itself, usually by adding Bt genes such that the plant can make pesticidal proteins (Cry1Ab, Cry1Ac, Cry1C, Cry1F, Cry2A, Cry3B and Cry9C) (*11, 12*) or to make the plant resistant to herbicides

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such as glyphosate (*13*). This enables the grower to apply herbicides in a more targeted and efficient manner without harming the crop. Presently, real-time PCR is the most widely applied technique for the quantification of genetically modified organisms in foods and feeds (*14*). Traditional PCR for detecting genetically modified crops (*15*) has high sensitivity and specificity, but only one target gene can be detected in most cases. Multiplex PCR methods can be used for detecting more than one target sequence, such as CaMV 35S-P, nos-P, nos-t, npt II, and epsps in soybean and tobacco (*16*). Enzyme-linked immunosorbent assays (ELISAs) have also been used to detect GMO proteins (*17, 18*), however, ELISA's can only detect one analyte at a time. Commercially available lateral flow immunochromatographic dipsticks have been developed for both single and multianalyte detection of GMOs (e.g., QuickStix™ Kit for Roundup Ready® Grain - AS 010 BG and QuickStix™ Combo Comb Kit for Cry1Ac/Cry2A/Roundup Ready® - AS 046 STC, EnviroLogix). Single and multianalyte dipsticks, while convenient, still yield mostly qualitative yes/no results.

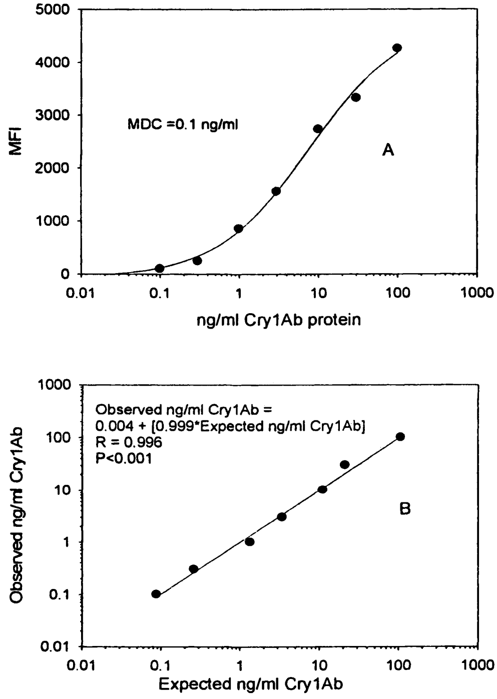

*Figure 1. Panel A- Fluorescent covalent microsphere immunoassay 4-PL plot of ng/mL Cry1Ab vs. median fluorescence intensity. Panel B- Linear regression of the results of observed vs. expected MFIs from 4-PL fit.*

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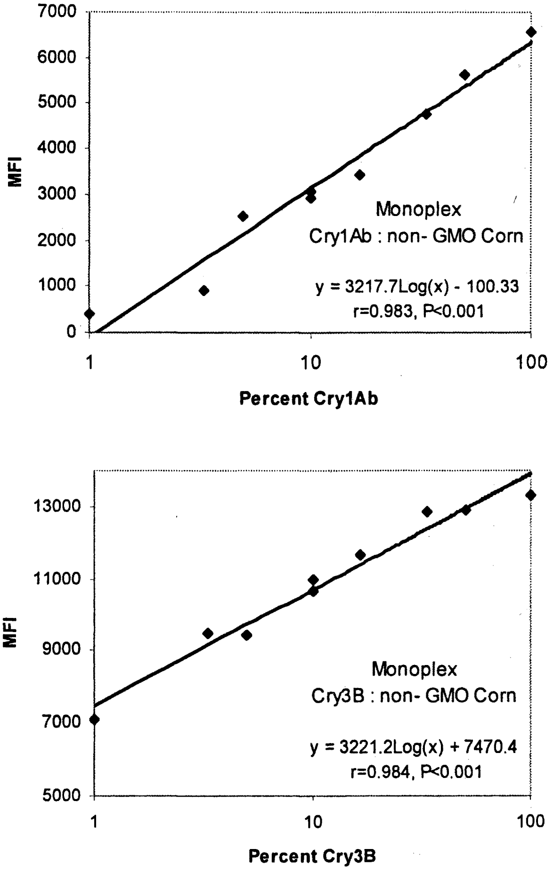

*Figure 2. Linear regression of monoplexed fluorescent covalent microsphere immunoassay vs. percent Cry1Ab (Panel A) or Cry3B (Panel B) extracted from non-GMO:GMO corn mixtures.*

The FCMIA could easily detect 1% GMO:non-GMO mixtures (the equivalent of 1 GMO kernel of Cry1Ab or Cry3B in 100 non-GMO kernels). One percent was the lowest ratio of GMO:non-GMO corn we used, however, the assay still had 1000s of MFI units before it would be expected to level off. This is supported by the demonstration of an MDC of 0.1 ng/mL for pure Cry1Ab.

Bt is now the most widely used biologically produced pest control agent. In 1995, worldwide sales of Bt were projected at $90 million, representing about 2% of the total global insecticide market with a worldwide distribution of 2.3 x $10^6$ kg (*19*). As of early 1998, there were nearly 200 registered Bt products in the United States (*19*). While the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favor the future development of Bt for the control of pests in forestry, and the control of mosquitoes and blackflies (*19*). Therefore rapid analytical methods are needed.

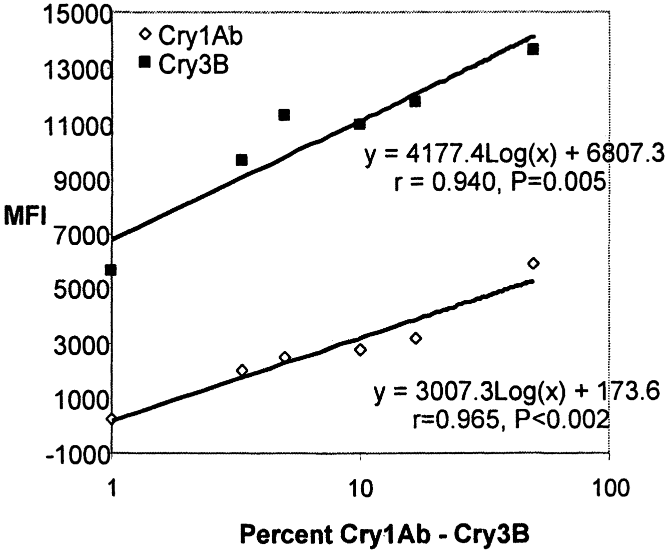

*Figure 3. Linear regression of diplexed fluorescent covalent microsphere immunoassays vs. percent Cry1Ab and Cry3B extracted from non-GMO:GMO corn mixtures.*

Multiplexed FCMIA technology has been shown to be useful in the measurement of diverse analytes such as cytokines (*20, 21*), cancer markers (*22*) gene expression (*23*), and markers of exposure to bioterrorism agents (*4, 6, 8*) and pesticides (*7*), etc. FCMIA has been shown to be more sensitive, faster and cheaper than alternative ELISA or chemical methodologies, and also has the capacity for remarkable throughput. Utilizing as few as 5 microplates, we have described an FCMIA, which has the capability of performing over 12,000 separate analyses in under 6 h (*5*).

In the present experiment we developed monoplex and diplex indirect sandwich FCMIAs for Cry1Ab and Cry3B extracted from GMO:non-GMO corn mixtures as well as a monoplex FCMIA for purified Cry1Ab. The monoplex FCMIA for Cry1Ab yielded a highly significant linear relationship when the observed vs. expected concentrations were calculated from the 4-PL fit. The mean recovery was 101.7 ± 19.8% (SD) over the concentration range of 0.1–100

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ng/mL Cry1Ab while 6 of the 7 Cry1Ab dilutions added to construct the curve were in the 70–130% range considered acceptable for these types of assay (*10*). The Cry1Ab MDC was 0.1 ng/mL. When extracts from pre-mixed GMO:non-GMO corn were evaluated for their Cry1Ab and Cry3B content highly linear relationships were observed on dilution, both in monoplex and diplex formats. Performing the FCMIA assay in diplex vs. a monoplex format showed no significant differences in MFI suggesting no cross-reactivity between Cry1Ab and Cry3B.

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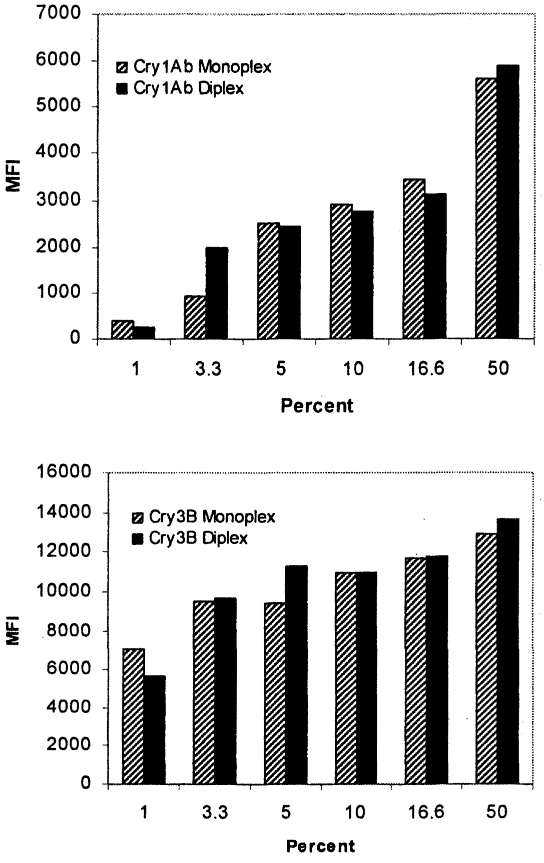

*Figure 4. Comparison of monoplexed vs. diplexed fluorescent covalent microsphere immunoassays for Cry1Ab and Cry3B extracted from non-GMO:GMO corn mixtures. One way repeated measures ANOVA Cry1Ab (Panel A), P=.602. One way repeated measures ANOVA Cry3B (Panel B), P=.588.*

In conclusion, we have shown proof of principle that Bt pesticidal proteins can be analyzed by multiplexed FCMIA.

## Acknowledgements

Mention of a product or company name does not constitute endorsement by NIOSH. This work was supported by an interagency agreement between NIOSH and NIEHS (Y02ES10189). The content and conclusions of this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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## Chapter 18

# Carbamate Pesticides and Their Biological Degradation: Prospects for Enzymatic Bioremediation

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Diverse carbamate compounds are widely used as insecticides, herbicides and fungicides in agriculture around the world. Environmental and human health concerns about off-target and off-site residues of various pesticides have sparked interest in the development of technologies for their detoxification. One promising technology for this purpose is enzymatic bioremediation, which uses formulations of detoxifying enzymes to clean up residues in contaminated environments. Here we review the feasibility of developing carbamate detoxifying enzymes as bioremediation agents.

Chemical pesticides have become indispensable in modern agriculture for controlling insect, plant and fungal pests. It has been estimated that, worldwide, 4 million tonnes of pesticides were applied to crops in 1999 (*1*). Global pesticide sales reached approximately US$33 billion in 2004 (*2*). However, the vast majority of the active ingredients does not reach target pests and some of it enters groundwater and rivers through misapplication, runoff and leaching (*3*). Accordingly, there is mounting public concern about the deleterious effects of

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pesticide contamination, through its impacts on both the environment and on human health. Technologies for decontaminating pesticide residues are therefore needed. Traditional methods of remediation of toxic compounds including incineration, burial or chemical degradation (oxidation, reduction and hydrolysis) are often too expensive or otherwise impractical for cleaning up the pesticide residues (*4*), but these problems may be ameliorated by bioremediation, a process by which biological agents and processes are utilised to detoxify environmental pollutants (*5-8*). Bioremediation has been applied effectively to the clean-up of pesticides in irrigation tailwater, groundwater and soil (*1*, *8-10*).

The direct application of live microorganisms to contaminated soils has been used to degrade a number of pesticides (*11-16*). Such microbes have evolved naturally or have been genetically engineered *in vitro* to metabolize contaminants to products that possess significantly reduced toxicological properties (*17-22*). However, this form of bioremediation is only applicable to some situations because of its reliance on microbial growth within the contaminated environment. Growth is often retarded by poor soil aeration and nutrition, leading to slow biodegradation rates that require weeks or months in order for desirable pesticide detoxification levels to be achieved. Additionally, the dependence on microbial growth precludes use in the rapid clean-up of moving water (e.g. irrigation run-off) or surface-contaminated commodities (e.g. fruit and vegetables).

An alternative to microbial remediation is the use of a relatively new technology known as enzymatic bioremediation. It is particularly suited to environments not conducive to microbial growth or situations where rapid remediation is required. This includes irrigation run-off water, spills, commodity clean-up, wash-down of farm machinery and for the personal protection of agricultural workers. Enzyme-based bioremediation often utilises the degradative capabilities of pesticide-resistant microorganisms, insects or weeds as a source of catalytic proteins (*23-28*). Typically, once an organism possessing the desired degradative property is isolated, gene technology is then used to clone the gene(s) responsible (*29-31*). The enzymatic properties of the resultant gene products are determined and, if necessary, improved using modern molecular biology techniques. These enzymes are then produced in quantity and applied directly to the affected area (*32-35*). This technology is currently being applied to the clean-up of pesticide residues from agricultural wastewater (*8*, *36*, *37*).

For enzymatic bioremediation to be feasible the enzyme must be stable *ex vivo*, have high substrate affinity (a low micromolar $K_m$), a $k_{cat}$ exceeding 100 turnovers per minute, and be cofactor-independent. Furthermore, enzyme performance must not decline significantly under such environmental stressors as fluctuating pH, temperature and ionic strength. Ideally, the enzyme should be capable of detoxifying a large group of chemically related pesticides to

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substantially less toxic metabolites. Furthermore, the production of the enzyme on a mass scale must be economically feasible (*34-37*).

Our laboratory at CSIRO, together with Orica Limited, has successfully implemented an enzymatic bioremediation technology for the clean-up of the organophosphate (OP) class of insecticides. We isolated an *Agrobacterium radiobacter* P230 organism that was capable of hydrolysing the phosphoester bond of aromatic oxon and thion OPs (*38*). The OP-degrading enzyme (named OpdA) isolated from this bacterial strain was produced in large scale using fermentation techniques by Orica, who then showed that it could decontaminate OP residues *in situ* under a wide range of environmental conditions (*8*). The product is now commercially available as LandGuard™.

Naturally, the application of this type of technology to other classes of pesticides is desirable. One such class of pesticide, the carbamates, is a structurally diverse group of compounds that have a widespread agricultural use as insecticidal, herbicidal and fungicidal agents. Here we review the microorganisms and microbial gene-enzyme systems involved in the biodegradation of carbamate pesticides, focusing in particular on the insecticidal and herbicidal carbamates.

## Types of Carbamate Compounds

Carbamate pesticides are derived from carbamic acid (HOOC-NH2) and possess the general structure shown in Figure 1. The side chains principally govern the biological activity of the pesticide. The atom denoted by the X is either an oxygen or a sulfur, whereas $R_1$ and $R_2$ can be a number of different organic side chains, although quite often a methyl group or a hydrogen. $R_3$ is usually a bulky aromatic group or an oxime moiety.

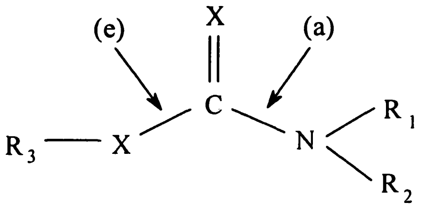

*Figure 1. General structure of carbamate compounds. X = O or S; $R_1$, $R_2$ and $R_3$ are usually organic side chains, although $R_1$ or $R_2$ may be a hydrogen (see text). The arrows indicate esterase (e) and amidase (a) cleavage sites.*

## Insecticidal Carbamates

Insecticidal carbamates (Figures 2A and 2B) fall into one of three types:

- aryl *N*-methyl carbamates (e.g. carbaryl, propoxur),
- *N*-methyl or *N*-dimethyl heterocyclic carbamates (e.g. carbofuran, pirimicarb), and
- *N*-oxime carbamates (e.g. aldicarb, methomyl).

The insecticidal activity of all three types arises from the inhibition of acetylcholinesterase (AChE), a mode of action that is effected through modification of an active site serine residue within the enzyme. The resultant carbamylation of this residue causes the accumulation of high concentrations of acetylcholine (ACh) within synapses. Although the reaction is reversible, AChE decarbamylation is relatively slow and may take up to 40 minutes (*39*). Nerve impulse transmission is thus affected and the continuous stimulation of muscle by ACh causes uncontrolled, rapid muscle movement, paralysis, convulsions and death. The insecticidal carbamates are moderately to highly toxic to mammals (*40*, *41*), however their hydrolysis yields metabolites that are generally more polar, soluble and somewhat less toxic than the parent molecule (*42*, *43*). In many cases, the environmental stability of these metabolites is also significantly lower than the parent pesticide compound (*44*).

## Herbicidal Carbamates

Herbicidal carbamates (Figures 2C and 2D) also fall into three groups:

- the phenyl carbamates (e.g. phenmedipham, desmedipham and chlorbufan),
- thiocarbamates (e.g. thiobencarb, molinate and EPTC), and
- dithiocarbamates (e.g. dazomet and metam, which are also fungicidal).

The thio- and dithiocarbamates differ structurally from the other carbamates, in having one or two sulfur groups as part of the carbamate ester linkage, respectively (*45*). The herbicidal carbamates possess a broad spectrum of biological activities and modes of action ranging from those that interfere with photosynthesis (phenmedipham and desmedipham), lipid synthesis inhibitors (molinate and EPTC) and non-selective enzyme inhibitors (dazomet) (*46*). In general, the herbicidal carbamates are of low mammalian toxicity and their metabolites are generally less toxic (and more reactive) than the parent compound (*47*).

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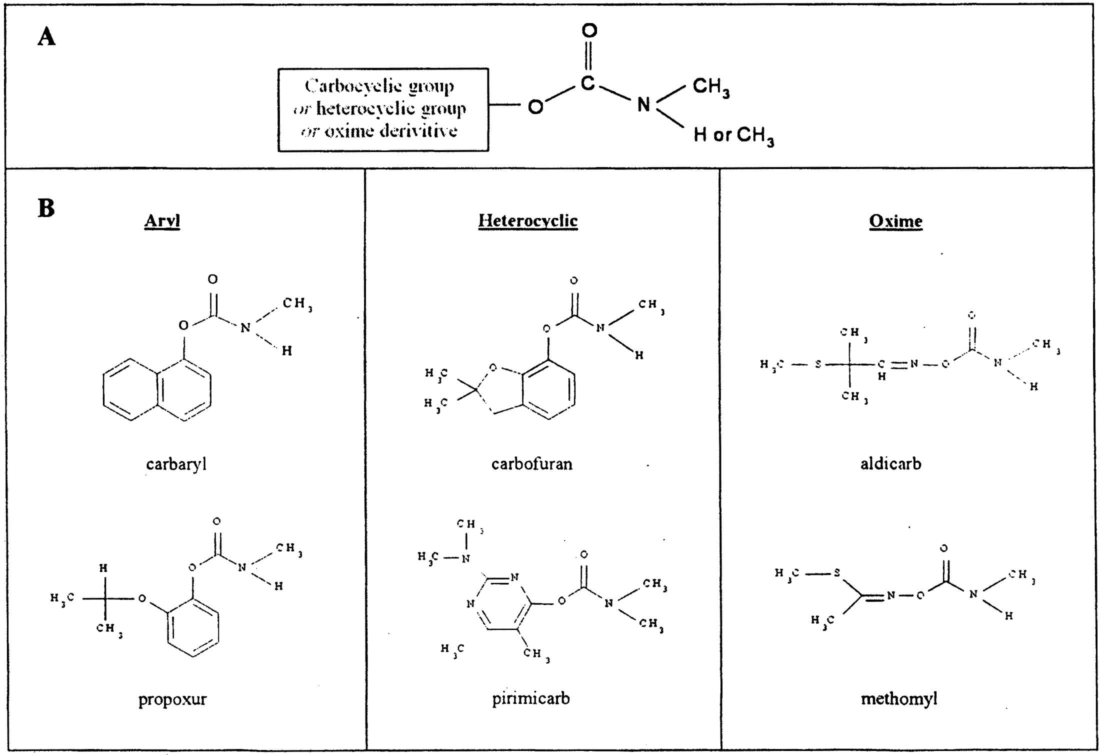
**Insecticidal carbamates**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aldicarb
propoxur
pirimicarb
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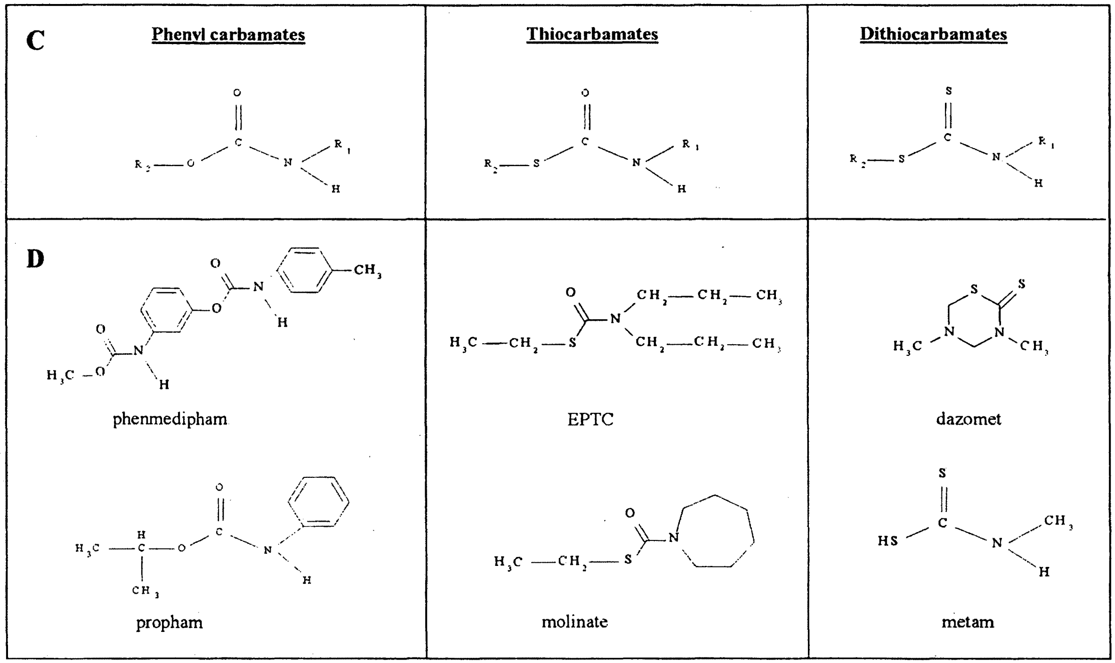
**Herbicidal carbamates**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Phenyl carbamates
Thiocarbamates
Dithiocarbamates
D
phenmedipham
EPTC
dazomet
propham
molinate
metam

*Figure 2. General structures (A, C) and specific examples (B, D) of insecticidal and herbicidal carbamates, respectively. In panel C, $R_1$ and $R_2$ are alkyl or aryl groups (for phenyl carbamates, $R_1$ and/or $R_2$ are phenyl groups).*

## Carbamate-Degrading Enzymes

Early studies provided evidence that microorganisms played an important role in enhancing carbamate degradation in "aggressive" soils in which repeated pesticide applications led to a greatly reduced persistence of these compounds (*48-52*). It has since been shown that the primary step in the carbamate degradation process is often hydrolysis across the carbamate linkage (*53*). This simple reaction is predominantly a cofactor-independent process and thus advantageous for enzymes that are to be considered for bioremediation. Indeed, several carbamate hydrolase enzymes responsible for the degradation have now been isolated from various organisms.

Many of the enzymes that hydrolyse carbamate compounds are either esterases or amidases. Esterases attack the C-O bond adjacent to the carbonyl group, whereas amidases hydrolyse the N-C bond next to this group (Figure 1). The reaction mechanism of the esterases and amidases is similar in that they require nucleophilic attack at the carbonyl carbon atom (*54*). Carbamate hydrolysis is also heavily influenced by the chemical structure of the side chains, and the substrate range of the carbamate hydrolases characterised to date has generally been quite limited. The parent compound is often substantively detoxified by these hydrolytic processes. For insecticidal carbamates, enzyme-mediated hydrolysis typically yields an alcohol as well as methylamine and carbon dioxide (*55*). While enzymatic hydrolysis has been reported for some herbicidal carbamates (*56*), no enzyme for fungicidal carbamate hydrolysis has been identified to date, although some of the degradation products produced from their *in vitro* and *in vivo* hydrolysis are well known (*57, 58*).

## Biological Degradation of Insecticidal Carbamates

### Characterized Genes

#### *MCD*

Karns *et al.* (*59*) isolated a single bacterial strain (termed WM111) capable of growth in minimal media that was supplemented with carbofuran as a sole source of nitrogen. The organism was identified as an *Achromobacter* sp. and could deplete 200 μg/ml of carbofuran by more than 100-fold within a 42-hour time period. Cell-free extracts fractionated from these cultures were found to hydrolyse the carbamate linkage of carbofuran to generate the 7-phenol metabolite (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate),

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thereby utilizing the nitrogen portion of the carbamate side chain. The native enzyme was purified and shown to hydrolyse carbofuran, carbaryl, aldicarb and o-nitrophenyl dimethylcarbamate (*60*). Low $K_m$ (15 and 56 μM) and high $V_{max}$ values (780 and 810 nmol min$^{-1}$ mg$^{-1}$) were determined for carbaryl and carbofuran, respectively, while a high $K_m$ (2800 μM) and a relatively lower $V_{max}$ value were observed with aldicarb. The $k_{cat}$ determinations for these three substrates is therefore in the range of 0.4 – 1.0 s$^{-1}$. Further characterisation of the enzyme confirmed that it exists as a dimer in its native form and requires $Mn^{2+}$ for enzyme activity (*61*), although the precise role of this cation in catalysis has not since been determined.

The plasmid-borne gene encoding the protein was identified (*62*) and named *mcd* (methylcarbamate degradation). The structural gene is ~2.0 kb and its sequence has been deposited in Genbank (accession no. AF160188). It has since been detected in a diverse array of carbofuran-degrading bacteria (*63-65*). Amino acid sequence analyses have shown that the enzyme is composed of two-fold repetitive units, both of which exhibit similarity to the members of the zinc metallo-hydrolase family (*66*). Interestingly, attempts by Karns *et al.* (*61*) to produce the MCD enzyme in heterologous systems including *Pseudomonas, E. coli* and other gram-negative bacterial hosts proved largely unsuccessful. Expression of the protein from a recombinant *E. coli* MCD plasmid harbouring an inducible upstream *lac* promoter also failed to yield the active hydrolase. Significant efforts might be required to engineer a system that could readily generate large quantities of the active protein for biochemical and biophysical studies, and consequently for enzymatic bioremediation purposes.

### *cahA*

An *Arthrobacter* sp. designated strain RC100 was isolated from agricultural soils exposed to repeated applications of carbaryl (*67*). Although the bacterium harboured three plasmids, one of these (pRC1) provided the microorganism with the ability to hydrolyse carbaryl directly to 1-naphthol, and DNA sequencing of the plasmid revealed a gene of approximately 1.5 kb in length, which was named *cahA* (Genbank accession no. AB081302). The native protein purified from *Arthrobacter* RC100 cell extracts (*68*) is ~51 kDa in size, is a homodimer, and metabolises carbaryl to α-naphthol ($K_m$ = 23 μM; $V_{max}$ = 210 nmol min$^{-1}$ mg$^{-1}$; $k_{cat}$ = 0.2 s$^{-1}$). The enzyme is not inhibited by EDTA and thus a metal cofactor requirement is unlikely. However, activity is dramatically reduced by serine inactivating agents such as PMSF and paraoxon, suggesting that serine is vitally involved in catalysis and is present within the enzyme active site. Indeed, the CahA protein possesses sequence similarity to the amidase signature enzymes (*69*) whose catalysis is mediated via conserved serine and lysine residues that

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comprise a catalytic dyad (*70*). The enzyme is active towards a number of aryl *N*-methyl carbamates other than carbaryl (e.g. metalocarb and xylylcarb), however it lacks affinity for other carbamates within this subgroup that contain different alkyl substitutions on the aromatic ring (e.g. propoxur and fenobucarb). No activity has been reported for heterocyclic or *N*-oxime carbamate derivatives. This narrow substrate specificity would no doubt reduce the enzyme's versatility as a potential bioremediation agent and protein engineering may be required to broaden the enzyme's metabolic capabilities. Helpfully, however, CahA has recently been overexpressed in *E. coli* (*71*), demonstrating a capacity for the active protein to be produced in this facile heterologous system.

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### *cehA*

A bacterial strain isolated from carbaryl-treated soil capable of metabolizing carbaryl to α-naphthol was identified as a *Rhizobium* species and was named strain AC100 (*72*). The gene coding for the carbaryl-degrading activity (found on a 25 kb plasmid) was subsequently cloned and named *cehA* (accession no. AB069723). The protein purified from *Rhizobium* sp. AC100 cultures is an 82 kDa dimer, and is not inactivated by serine hydrolase inhibitors or EDTA. Interestingly, there are no homologous sequences in the molecular databases even though the enzyme possesses hydrolase activity towards numerous aryl *N*-methyl carbamates (with a $K_m$ value for carbaryl of 62 μM). No activity was detected towards carbofuran. The purified enzyme hydrolyses 1-naphthyl acetate and 4-nitrophenyl acetate, suggesting that it is an esterase, however the cehA protein sequence does not possess the classic esterase sequence motifs (*73*) and so it remains unclear as to whether cehA acts at the carboxylester or the amide carbamate linkage. More study will be required on the kinetic parameters of this enzyme to determine its suitability as a bioremediation enzyme.

## Other Microbial Systems and Enzymes

A number of other organisms capable of degrading insecticidal carbamates have been isolated, however the gene(s) responsible for this property have not been identified to date. *Pseudomonas* strains feature predominantly in this list. Soil enrichment cultures containing carbaryl as the sole carbon and energy source yielded a Pseudomonad strain named CRL-OK from which a dimeric, 187 kDa cytoplasmic protein consisting of two identical subunits was purified (*74*). The enzyme is active towards carbaryl, carbofuran and aldicarb, but with moderately high $K_m$ values for each pesticide (213, 140 and 542 μM, respectively). A *Pseudomonas aeruginosa* sp. 50581, capable of metabolising carbaryl to 1-naphthol, was purified from soil (*75*) and the monomeric, 65 kDa enzyme purified from cultures remains as the only membrane-bound carbaryl hydrolase reported in the literature (*76*). The protein has a very narrow substrate specificity, and was unable to degrade *N*-methyl carbamate compounds other than carbaryl, however its high affinity for this substrate ($K_m$ = 9 μM) and excellent turnover value ($V_{max}$ = 7900 nmol min$^{-1}$ mg$^{-1}$) would make it a very attractive candidate for carbaryl bioremediation. Another strain, *Pseudomonas* sp. 50432, isolated from carbofuran-treated agricultural soils (*77*) was later shown to contain a cytoplasmic, constitutively-expressed enzyme that was capable of metabolizing carbofuran via hydrolytic and oxidative pathways (*78*). The purified native protein was found to be cofactor-independent, monomeric and active towards carbaryl, carbofuran, and aldicarb, with low $K_m$ (16 and 12 μM) and high turnover values (1600 and 1725 nmol min$^{-1}$ mg$^{-1}$) reported for carbaryl and carbofuran, respectively (*79*). This ability of this enzyme to metabolise several pesticides with high affinity and catalytic efficiency suggest that it may be highly useful in the detoxification of soils that have been exposed to a cocktail of insecticidal pesticide compounds. More recently, carbaryl-degrading *Pseudomonas* strains (sp. C4, C5 and C6) have been isolated from garden soil samples and characterized metabolically (*80*) however the gene-enzyme systems involved have not yet been identified to date.

One of the most interesting studies on carbamate degrading organisms has been a 2003 report of a carbaryl hydrolase enzyme isolated from the fungus *Aspergillus niger* (*81*). Carbaryl-treated soil from a vegetable farm in China was inoculated into soil enrichment cultures and maintained on media containing 1% carbaryl as the sole carbon source. A fungal strain, designated PY168, demonstrated good growth and cell extracts exhibited carbaryl hydrolytic activity. The enzyme responsible for this activity was purified and shown to be a ~50 kDa monomeric protein that was stable across a broad pH (5-8.5) and temperature (15-50°C) range. It is inhibited by serine-inactivating agents but not EDTA, indicative of an active site serine within the enzyme but not a metal cofactor. The highlight of this study was the impressive metabolic properties of the enzyme; not only was it active towards carbamate pesticides spanning a large substrate range (which included carbaryl, carbofuran and aldicarb), but the kinetic parameters were far superior to those of previous studies in that much higher turnover values were observed when the enzyme was tested against 11 different carbamate compounds (*81*). Indeed, the $k_{cat}$ values for the substrates tested (670 – 1724 s$^{-1}$) towards the *A. niger* enzyme were found to be two to three orders of magnitude greater than those reported for the bacterial carbaryl hydrolases (*61*, *68*, *74*, *76*, *79*). Isolation of the gene encoding this promising enzyme is clearly now a priority.

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# Biological Degradation of Herbicidal Carbamates

## Characterized Genes

### *PCD*

*Arthrobacter oxydans* P52, isolated from soil enrichment cultures, was found to degrade the bisphenylcarbamate herbicides phenmedipham and desmedipham via hydrolysis of their central carbamate linkage (*56*). From this strain a gene, *pcd*, encoding a phenyl carbamate hydrolase (PCD) was cloned and sequenced (accession no. M94965). The native PCD enzyme was shown to be a 55 kDa monomer with respectable turnover values for phenmedipham and desmedipham ($V_{max}$ values of 170 and 71 nmol min$^{-1}$ mg$^{-1}$, respectively). No activity was observed for phenisopham, however, which differs only in the presence of an alkyl substituent at the amide group. PCD also hydrolyses chlorpropham, which possesses an aryl group on the amide side of the carbamate linkage and an alkyl group on the carboxyl ester side, but at a rate 100 times slower than that observed for the bisphenylcarbamates. PCD is also active on the esterase substrate *p*-nitrophenylbutyrate ($K_m$ = 25 μM), and indeed shares homology with esterases of eukaryotic origin (*82*). Since its carbamate activity appears restricted mostly to phenyl carbamate herbicides, an improvement of the PCD enzyme for remediation purposes would probably be warranted.

### *Cytochrome P450 System*

The degradation of EPTC (*S*-ethyl dipropylcarbamothioate) by *Rhodococcus* sp. strain N186/21 (*83*) was found to involve an inducible cytochrome P450 system and an aldehyde dehydrogenase (*84*). EPTC metabolism was proposed to be catalysed initially by a cytochrome P450 monooxygenase, where the α-propyl carbon of the *N,N*-dialkyl moiety undergoes a hydroxylation to produce the unstable metabolite α-hydroxypropyl EPTC, which would decompose into propionaldehyde and *N*-depropyl EPTC (*84*). The released aldehyde was then further metabolised into the corresponding carboxylic acid by aldehyde dehydrogenase. It was later shown that the cytochrome P-450 system was encoded by three genes, *thcB* (cytochrome P-450 monooxygenase), *thcC* (2Fe-2S ferredoxin), and *thcD* (ferredoxin reductase), all of which are required for the degradative phenotype (accession no. U17130). Clearly, adapting a system such as this for *ex vivo* use in bioremediation would be fairly problematic. The requirement for metals or metal-containing cofactors (e.g. iron or heme prosthetic group) for ferredoxin reductase and cytochrome P450 enzyme activity is one obvious problem. Limitations also arise due to the

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need for coenzymes such as endogenous P450 reductase and cytochrome $b_5$ to facilitate catalysis by the heterologously expressed cytochrome P450 enzyme (*85*). Advances in crop biotechnology (*86*, *87*) have led to the development of transgenic, herbicide-resistant crops co-expressing P450 enzymes that metabolize various herbicidal compounds *in vivo* (*88-90*). However, despite an ongoing interest in P450 enzymes as biodegradation agents (*91*, *92*), the direct application of functional cytochrome P450s in a free enzyme bioremediation approach has not been undertaken.

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## Other Microbial Systems and Enzymes

Soil microorganisms contribute significantly to thiocarbamate degradation, although identification of the microbes and enzymes involved and modes of degradation have not been extensively reported (*93*). Research concerning the microbial metabolism of thiocarbamate compounds has been primarily conducted for the herbicides molinate and EPTC above. However, some of the pathways discussed could be easily applied to other members of the thiocarbamate group, including various oxidative transformations, dealkylations and thioester bond cleavage.

Various pathways have been proposed for the degradation of molinate based on studies examining the fate of molinate with mixed populations of microorganisms and pure cultures. These are oxidation of the azepine ring to form hydroxy- and oxo-molinate (*94-96*), oxidation of the sulfur atom to form molinate sulfoxide and sulfone (*95*, *97*), and oxidation of the ethyl moiety to produce molinate alcohol and acid (*95*). The predominant oxidation product of molinate, though, is the sulfoxide metabolite (*98*). This is of particular concern as this metabolite is in fact more persistent in soil and possesses a greater mammalian toxicity than molinate (*99*, *100*).

The enzymes involved in the degradation of molinate and the oxidised metabolites are generally not well understood, although a novel pathway for molinate degradation by a defined bacterial mixed culture has recently been elucidated (*101*). The initial step in the pathway was carried out by an actinomycete strain, which cleaved the thioester bond of molinate resulting in the accumulation of hexamethyleneimine and ethanethiol and the spontaneous conversion of ethanethiol to diethyl disulfide. Hexamethyleneimine and the sulfur containing metabolites were further metabolised by other species in the mixed culture so that molinate was completely mineralised.

The microbial metabolism of EPTC has been characterized for a host of microorganisms, including *Rhodococcus* sp. (*83*), *Arthrobacter* sp. (*102*) and *Bacillus* sp. (*103*). A *Rhodococcus* sp. strain NI86/21 utilises EPTC as the sole carbon and nitrogen source, and degradation is proposed to occur initially through *N*-dealkylation, followed by conversion of the released aldehyde into the corresponding carboxylic acid (*83*). The strain was also capable of growth with thiocarbamate compounds vernolate, butylate and to a lesser extent cycloate. However, no growth was observed with molinate. An alternative pathway for EPTC metabolism in the genus *Rhodococcus* was proposed by McClung *et al.* (*104*) for the hydrolysis of EPTC across the thioester bond to release ethanethiol, dipropilamine and $CO_2$.

A bacterial strain possessing thiobencarb degradative properties was isolated from soil perfused with a thiobencarb solution and identified as a *Corynebacterium* sp. (*105*). Thiobencarb degradative activity was encoded on a plasmid and loss of the plasmid led to loss of thiobencarb degradative activity. No further characterisation of this activity has been reported. Dechlorination of thiobencarb has also been observed under biological conditions. The metabolite produced from this reaction, S-benzyl *N,N*,-diethylthiocarbamate (de-chlorothiobencarb), causes dwarfing of rice plants (*106*). The causative microbes have been demonstrated to be facultative anaerobes (*107*). In the environment, thiobencarb is reported to undergo various pathways of transformation, including oxidation at the benzene ring, oxidation at the sulfur moiety, dealkylation, and cleavage of the thioester bond (*47*).

There are very few dithiocarbamate herbicides currently in agricultural use and thus the data available reporting their degradation are limited. Ibekwe et al. (*108*) described the enrichment of soil microbial communities capable of degrading metam-sodium from agricultural soils with a history of exposure. However, no pathways of degradation or specific enzymes were described.

## Conclusions

Enzymatic bioremediation is a technology designed to decontaminate pesticide residues found in agricultural soils and wastewater, and on post-harvest fruits and vegetables. Modern molecular biology techniques are used to isolate the gene-enzyme systems from microorganisms harbouring the desired degradative processes, and in many cases various biotechnologies are used to improve the enzymatic catalytic capabilities (*109*). While industrial applications have been successfully implemented for enzymes degrading various classes of OP insecticides, no such remediatory system is currently available for the carbamate pesticides. A number of gene-enzyme systems have been characterized, however, particularly for insecticidal and herbicidal carbamate biodegradation, although in many cases the enzyme properties require improvement in order to meet the performance criteria for commercial use and to facilitate their production in large quantities. In the case of fungicidal carbamates, gene-enzyme systems involved in their metabolism are yet to be

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identified. Nonetheless, industrially applied biological clean-up technologies for each class of the carbamate pesticides discussed in this review are feasible. Moreover, they should pose great potential once the challenges of gene isolation and enhanced capabilities and production of the recombinant carbamate-degrading enzymes have been fully addressed.

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## Chapter 19

# Antifungal Antibodies in Plant Pathology

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For several decades, polyclonal and monoclonal antibodies, and more recently recombinant antibodies and fragments thereof have been used for pesticide residue analysis and biomedical applications. Antibodies are being used now to treat cancer, and have been produced in plants for protection against viral and fungal pathogens, as well as for pesticide resistance. This article comprises a review of anti-fungal monoclonal, polyclonal and recombinant antibodies used in plant pathology over the last two decades. The antigens used for animal immunizations, the specificity of the antibodies thereby obtained, and a few examples of practical applications of these antibodies such as immunodiagnostics, immunofluorescence, fungal growth inhibition, aerobiology and glycobiology studies are reviewed.

## Introduction

In plant pathology, immunological methods have been mainly used to detect or identify plant viruses and fungi (*1*). One of the greatest hurdles to further use of anti-fungal antibodies in plant pathology has been their lack of specificity (*1, 2*). Yet, phytopathogenic fungi are responsible for extensive losses in food, food storage and food distribution (*3*). Moreover, the presence of fungi can seriously affect the nutritional value, colour, flavour and texture of grains and plant products for example, and the production of mycotoxins may pose a health risk to human populations (*2*).

Antibodies to a great variety of fungi and/or fungal antigens have been raised, most often, to develop enzyme-linked immunosorbent assays (*1,4,5,6,7,8,9,10,11,12*), especially for the detection of molds (*13,14,15,16,17*). However, the elucidation of taxonomic relationships among fungal species (*5*), the identification of common structures in different fungal species (*18,19*), disease prediction modelling and aerobiology (*20,21*) as well as the understanding of fungal biology, glycobiology, morphology and host/pathogen interactions at the molecular level (*22*) are other applications of antibodies in plant pathology. A comprehensive list of polyclonal and monoclonal antibodies against plant pathogenic fungi are given in Tables I and II, along with the antigens used for immunizations, the antibody specificity and application.

Each type of antibody (polyclonal, monoclonal, recombinant) has its own advantages and disadvantages: Polyclonal antibodies are easy to produce, but possess multiple specificities and are not reproducible. Monoclonal antibodies on the other hand, are issued from a single clone with one unique specificity. However, they require expertise and are costly to produce. Recombinant antibodies finally, are isolated along with their respective genes and can therefore be easily manipulated. However, they tend to be, especially in the case of antibody fragments, less stable than their parental, full-size counterparts.

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## Fungal Antigens for Animal Immunizations

Fungal immunogens used to raise antibodies have been described in several excellent review articles (*1,4,5*) as well as fungal antigen preparation (*12,23,24,25*) and their binding to microtiter plate wells for immunoassay applications (*13*). A variety of fungal materials have been used for the production of fungal-specific antibodies, among which the most commonly used are mycelium and mycelium-derived immunogens. Others are zoospores, spores, conidia, ascospores, cysts, as well as glycoproteins, exoantigens and polysaccharides.

## Anti-fungal Antibodies Specificity and Affinity

Antibody specificity has been the greatest hurdle in the past to further use of antibodies in plant pathology. This has to be circumvented to make ELISA amenable to routine analysis of fungal infection of plant material, food and other environmental matrices. Overall, antibodies raised against the fungal mycelium have lower specificity compared to antibodies raised against zoospores and cysts. Although such cross-specificity may be of some interest, in general higher specificity is required for the accurate detection and quantification of fungal pathogens or antigens.

Carbohydrate antigens seem to be fairly good immunogens, leading to specific antibodies, as high as race-specific in some cases (*69*). However, several fungi genera may display similar carbohydrate structures on their surfaces. This is the case with *Aspergillus* and *Penicillium* conidia and mycelia, which both display galactomannans at their cell surface (*70*). When a monoclonal antibody specific for galactomannan was tested for cross-reactivity to other fungi however, it showed very little cross-reactivity except for these two other genera (*70*). Several antibodies raised against fungi appear to bind to carbohydrate epitopes at the surface of these fungi (*70,71,72*) or to carbohydrate moieties specific to a layer of the fungi surface (*70*).

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## Use of Anti-fungal Antibodies in Plant Pathology

Antibodies possess several advantages over traditional methods and conventional assays to identify, quantify and neutralize fungal pathogens. One of these advantages is that antibodies can detect fungal pathogens even before the appearance of disease symptoms (*51*). Antibodies can be used for a wide range of applications in plant pathology: not only can they be useful in fungal immunodiagnostics, immunofluorescence and immunoblotting, but also in neutralizing fungal growth, as a tool to predict fungal infection as well as a tool to study fungal morphology, biology and reproduction. More recently, antibodies have even been expressed *in planta* for resistance against fungal diseases.

### Fungal Immunodiagnostics

Most antibodies raised against fungal antigens have been used to develop immunodiagnotic tools such as ELISA or other antibody-based assays. Routine methods for fungal detection are based on plate cultures, microscopy and chemical techniques, but these methods have low specificity and are time-consuming compared to immunotechniques (*37*). Immunodiagnostics of fungal pathogens are important, allowing for the detection of potentially harmful toxins in food as well as better disease management strategies.

Immunoassays to many different fungal pathogens have been developed: soil-borne and root-infecting fungi such as *Phytophthora fragariae* (*60*) and *Pythium ultimatum* (*73*), foliar fungi such as *Phytophthora infestans* (*61*), seedborne fungi such as *Pyrenophora* spp. (*74*), spores of airborne fungi such as *Pyricularia grisea* (*75*) as well as postharvest spoilage-causing fungi such as *Botrytis cinerea* (*33,61*), to give only a few examples (reviewed in 1).

## Immunofluorescence

Several anti-fungal polyclonal antibodies (*50,44*) as well as monoclonal antibodies (*72*) have been used in immunofluorescence studies. For example, polyclonal antibodies produced against the soluble and cell-wall antigens of *Phaeolus schweinitzii* (*50*) and *Mycena galopus* (*44*). *P. schweintzii* is responsible for root and butt-rot in a number of conifer species.

Polyclonal antibodies raised against the mycelium of *Fusarium oxysporum*, a fungus responsible for root rot in soybean, have been used to detect by immunoassay and locate by immunostaining common antigens shared by the host and pathogen (*42*). The antigens could not be identified, but were detected around the xylem elements, endodermis and epidermal cells of soybean's root sections, and also present on the mycelium, microconidia, macroconidia and chlamydospores of the fungus (*42*). A possible role of these common antigens as a basic factor in the compatibility between host and pathogen was investigated, since differential fluorescence in the resistant and susceptible cultivars examined was observed, as previous studies had suggested (*42*).

## Fungal Growth Inhibition and Plant Protection

Three monoclonal antibodies have been produced against the endophytic fungus *Neotyphodium ceonophialum* (*3*). These monoclonal antibodies were incorporated into fungal culture medium to determine their effect on fungal growth (*3*). These *Neotyphodium*-specific antibodies inhibited fungal growth, and microscopy revealed that upon binding of the monoclonal antibodies to the fungus, the latter changed its morphology with clumps and few hyphal growth (*3*). The monoclonal antibodies seem to bind specifically to a cell-wall bound component of the mycelium, but its function remains unknown (*3*).

Antibodies expressed in plants could protect the latter against various pathogens. Several antibodies have been expressed in plants already, a few of which targeted for plant protection against pathogens such as viruses (*76,77,78*) and mollicutes (*79*). In this work, three phage-display libraries were created with the mRNA from chickens immunized with three different *Fusarium graminearum* antigens: cell-wall bound proteins, mycelium surface proteins and germinated spores (*80*). The libraries were pooled and screened for the isolation of cell-wall specific antibodies over three rounds of panning (*80*). The recombinant antibody isolated was fused to one of three antifungal peptides, and

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**Table I: Polyclonal antibodies against plant pathogenic fungi**

| *Fungus* | *Antigen* | *Specificity* | *Usage* | *Reference* |
|---|---|---|---|---|
| Aspergillus spp. | M | + | Taxonomy | *18* |
| Alternaria spp. | M | + | IF detection | *26* |
| A. alternata | M | + | ELISA | *27,28* |
| A. ochraceus | ExAg | +++ | ELISA | *29,30* |
| A. versicolor | EPS | | ELISA | *31* |
| Botrytis spp. | M | + | Detection | *32* |
| Botrytis cinerea | M | + | Detection | *33* |
| Botrytis fabae | M | + | Immunocytochemistry | *34* |
| Cladosporium cladosporioides | EPS<br>M | +<br>+++ | Glycobiology | *35* |
| C. herbarum | EPS | ++ | ELISA | *31* |
| Fusarium spp. | M | + | ELISA | *36* |
| Fusarium graminearum & F. moniliforme | ExAg, M | ++ | ELISA, IB | *37* |
| F. moniliforme | M | + | ELISA | *40* |
| F. oxysporum | EPS | ++ | ELISA | *25* |
| F. oxysporum | EPS | ++ | Glycobiology | *25,38* |
| F. oxysporum | M | + | ELISA | *39* |
| F. oxysporum | Conidia | +++ | ELISA | *41* |
| F. oxysporum | M | ++ | ELISA, IF | *42* |
| F. poae | ExAg, M | +++ | ELISA, IB | *37* |
| F. sporotrichoides | ExAg | ++ | ELISA, IB | *37* |
| Gaeumannomyces candidum | EPS | ++ | ELISA | *31* |
| G. graminis | M | + | Detection | *43* |
| Macrophomina circinelloides | EPS | + | ELISA | *26* |
| M. phaseolina | M | + | ELISA | *32* |
| Monascus spp. | M | + | Detection | *32* |
| Mycena galopus | M | + | IF | *44* |
| Mycosphaerella brassicicola | Ascospore | + | Aerobiology | *20,21* |
| Ophiostoma ulmi | M | +++ | ELISA | *45* |
| P. aurantiogriseum | ExAg | +++ | ELISA | *46* |
| P. aurantiogriseum | M | + | ELISA | *14* |
| P. chrysogenum | EPS | ++ | ELISA | *31* |

**Table I:** ***Continued.***

| *Fungus* | *Antigen* | *Specificity* | *Usage* | *Reference* |
|---|---|---|---|---|
| P. digitatum | EPS | ++ | Detection assay | *47* |
| P. verrucosum | M | + | Taxonomy | *48* |
| Peronospora viciae | M | + | Detection | *49* |
| Phaeolus schweinitzii | M | +++ | IF | *50* |
| Phomopsis spp. | M | + | ELISA | *23,51,52* |
| Phytophthora spp. | M | ++ | Taxonomy | *53* |
| Phytophthora spp. | M | + | ELISA | *54* |
| Phytophthora spp. | M | + | ELISA | *55* |
| Phytophthora cinnamomi | zoospores | | Dip-stick assay and structural biology studies | *56,57,58,59* |
| P. fragariae | M | + | ELISA | *60* |
| Phytophthora infestans | M | + | Fungal biomass estimation | *61* |
| Plasmopara halstedii | M | + | ELISA | *61* |
| Postia placenta | β1-4 xylanase | + | Detection assay | *62* |
| Pyricularia oryzae | M | +++ | ELISA | *63* |
| Pythium violae | M | + | ELISA | *64* |
| Rhizoctonia solani | Toxin | +++ | ELISA | *65* |
| R. stolonifer | M | M | +++ | *27,28* |
| Sclerotinia sclerotiorum | ExAgs | ++ | ELISA | *66* |
| Spongospora subterranea | spores | + | ELISA | *67* |
| Tilletia indica | M | ++ | ELISA | *68* |

EPS : Extracellular polysaccharide
ExAg: exoantigens
M : Mycelium/mycelium-derived antigen(s)

+++: Species-specific
++: Genus-specific
+: Less than genus-specific

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expressed *in planta* (*80*). Expression of the antibody fused to any of the three antifungal peptides resulted in a high level of protection against *Fusarium oxysporum*due to interference with fungal growth *in planta* (*80*). Such results show that antibodies may prove an alternative for the production of transgenic crop plants resistant to fungal pathogens (*80*).

**Table II: Monoclonal antibodies against plant pathogenic fungi**

| *Fungus* | *Antigen* | *Specificity* | *Usage* | *Reference* |
|---|---|---|---|---|
| Botrytis cinerea | ExAg | ? | ELISA | *81* |
| Botrytis cinerea | conidia | ? | ELISA | *24,82* |
| Collecotrichum lindemuthianum | M | ? | Isolation | *83,84* |
| Fusarium oxysporum | M | + | Detection | *85* |
| Fusarium spp. | M | +++ | Detection? | *86* |
| Gliocladium roseum | M | +++ | colonization studies | *71* |
| Humicola lanuginosa | M | + | ELISA | *3,87* |
| Microdochium nivale | M | +++ | ? | *86* |
| Neotyphodium coenophialum | M | +++ | Growth control | *3* |
| Ophiostoma ulmi | M | +++ | ELISA | *2,45* |
| Penicillum aurantiogriseum | M | + | ELISA | *88* |
| P. bilaii | Galactomannan | + | ? | *89* |
| P. brevicompactum | Spores | + | Aerobiology | *90* |
| P. frequentans | EPS | +++ | ? | *70* |
| P. islandicum | ExAg | + | ELISA | *6,91,92* |
| Phytophtora cinnamomi | Zoospores, cysts | +++ | Surface component ID | *72* |
| P. megasperma | Glycoprotein |  | Detection | *93* |
| Plasmopara halstedii | M | +++ | ELISA | *94* |
| Postia placenta | B-1,4-xylanase | + | Capture assay | *62* |
| Pyrenophora graminea | ? | ? | ELISA | *74* |
| Pythium sulcatum | M | + | ELISA | *95* |
| P. ultimatum | ? | +++ | ELISA | *73* |
| Rhizoctonia solani | M | ? | IF, detection | *96,97* |
| Trichoderma | Glycoprotein | ++ | IF, detection | *98-102* |

| | |
|---|---|
| EPS : Extracellular polysaccharide | +++: Species-specific |
| ExAg: exoantigens | ++: Genus-specific |
| M : Mycelium/mycelium-derived antigen(s) | +: Less than genus-specific |

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### Aerobiology

Detection of airborne fungal spores represents a major challenge in plant pathology and epidemiology (*90,103*). Detecting the source and importance of spore inoculum in the field could be of great assistance in order to achieve efficient use of fungicides, both in terms of timing and quantities (*20*). Kennedy *et al.* (*20*) produced polyclonal antibodies against the ascospores of *Mycosphaerella brassisicola*, a fungus responsible for ringspot, a major foliar disease of vegetable brassicas. The antibodies were used in conjunction with an existing spore trapping system to detect ascospores by immunofluorescence (*20*).

Although relatively specific, the antibodies cross-reacted with the condia of several fungal species (*Pyrenopeziza brassicae*, *Mycosphaerella pinodes* and *Cladosporium herbarum*) and wall components of ascospores of *G. graminis*, *M. pinodes* and *P. brassicae* (*21*). Another method to monitor airborne inoculum was developed and investigated by Kennedy *et al.* (*21*). The system consisted in a microtiter immunospore trapping device where a suction system actively sucked air particles and impacted them onto microtiter plate wells. The system was tested with two fungal pathogens commonly found in brassica fields, *M. brassisicola* and *Botrytis cinerea*, and polyclonal antibodies developed against ascospores of *M. brassisicola* and conidia of *Botrytis cinerea* (*21*). Airborne inoculum could thereby be sampled in the field and targeted organisms rapidly and accurately quantified without any microscopic examination being required (*21*).

### Fungal Pathogen Biology, Glycobiology and Taxonomic Studies

Immunological techniques have been used to study several aspects of plant pathology such as biology, disease physiology (*34,65*) and fungal glycobiology (*19,22*). Knowledge gained from these studies can improve our understanding of some processes involved in fungal growth morphology (*104*), reproduction (*58*) as well as host/pathogen interactions (*68*). Polyclonal antibodies developed against mycelial antigens of *Tilletia indica* (causing agent of karnal bunt in wheat) were used to investigate differential expression of proteins or epitopes at the surface of the pathogen at different stages of its development (*68*). It was demonstrated that several epitopes were modulated during growth and sporulation of *T. indica* (*68*). The identification of epitopes specific to species and genera can be very useful in fungal taxonomy as well (*42,56,92*).

## Recombinant Antibodies

Recombinant antibody technology has been developed in the 1990s, and opens new possibilities of great interest for plant pathologists. Animal immunization is no longer necessarily required, antibodies against virtually any fungal antigen can be obtained in a few weeks from naïve libraries, or a few months from immune libraries, and these antibody fragments can be expressed in bacteria such as *Escherichia coli* to obtain an endless supply of consistent material. For a full review of recombinant antibody technology see *105,106*.

With recombinant antibody technology, antibodies and fragments thereof are displayed at the surface of filamentous bacteriophage (phage-display antibody libraries), yeast (yeast-display) or ribosomes (ribosome-display). The display technology allows us to select, or isolate, an antibody of interest from the library by a process generally referred to as "panning", where the antibody library is exposed to the antigen, and antibodies displayed which bind to this antigen can be isolated from the rest of the library and then expressed in bacteria, plants or yeast. The advantage of these display technologies is that the phenotype (the antibody protein) is linked to the genotype (antibody DNA), so that both are selected simultaneously. Moreover, stringent antibody selection strategies can isolate clones highly specific for a single fungal species, based on the panning strategy used. Panning only requires a few weeks (once the library has been constructed), and can result in antibody clones with greater affinity and specificity compared to those of polyclonal and monoclonal antibodies.

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## Conclusion

Polyclonal and monoclonal antibodies, and more recently recombinant antibodies have all been used in plant pathology. These antibodies have shown various degrees of specificity, depending mainly on the fungal immunogen used for animal immunizations: mycelium and mycelium-derived antigens, zoospores, ascospores, conidia, cysts, and fungal surface components such as glycoproteins, exoantigens and polysaccharides. The antibodies have been successfully used to develop immunodiagnostics and tools for fungal immunofluorescence, fungal identification, growth inhibition, disease prediction modelling and to study the plant pathogen's biology, reproduction and glycobiology. However, with the advent of recombinant antibody technology, where highly specific antibodies against any fungal plant pathogen or its components can be rapidly isolated, novel and exciting applications in plant protection and disease prediction can be foreseen.

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## Chapter 20

# Pesticide Risk Reduction by Management Practices: An Environmental Case Study of the Australian Cotton Industry

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The Australian Cotton Industry provides a valuable case study into rational methods for the selection and use of agrochemicals. The industry suffered heavy criticism and economic losses in the late 1990s because of pesticide drift issues and contamination of produce. Consequently, the industry invested in research on practices of agrochemical use by growers. Significant changes, documented in a 'best management practices' manual, included the introduction of genetically-modified cotton varieties, the education of growers into reducing the use of broad-spectrum sprays, and the promotion of on-farm wetlands as buffers to limit water-borne pesticide residues moving off-farm and reducing their persistence on-farm. This chapter draws together data resulting from these innovations and assesses the reduction of environmental risk they have afforded.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

## Introduction

The question of whether or not pesticides will be used depends on the perceived benefits versus the costs. Rather than being banned outright, useful chemicals with detrimental side effects can be replaced, restricted, or their use continued with management systems in place. The pattern of the continuing use of pesticides beneficial for Australian cotton production illustrates these possibilities. For example, the persistent insecticide DDT [50-29-3] was banned in Australia in 1987 and dieldrin [60-57-1] was banned in 1995 (*1,2*); more recently, chlorfluazuron [71422-67-8] was withdrawn from registration for cotton because of its demonstrated potential to contaminate beef (*3*). In contrast, the insecticide endosulfan [115-29-7], which was also found to contaminate beef (*4*), was not banned because of its efficacy in controlling the major cotton pest *Helicoverpa*. Instead its use has been restricted and conditions placed on its use to prevent contamination of beef. Meanwhile, the use of other registered pesticides continues with management systems in place to prevent off-site movement and environmental contamination.

This chapter draws on the available data from the Australian cotton industry to test the claims of reduction of environmental risk from the introduction of these practices. A set of hazard and risk assessments are presented to illustrate changes in hazard from the use of genetically modified cotton varieties. The data available for analysis are presented within the accepted risk assessment framework (*6*), whereby exposure is compared to effect by the use of a risk quotient and, where possible, the use of probabilistic analysis (*7*).

The focus of this chapter is at the farm level, to document exposures, with estimates of potential risk reductions by the various management practices. Although the data presented have been collected from a number of different sites and experiments conducted in cotton production systems, the results integrate the available technologies, introducing the concept of the 'farming parkland' as a future goal. The changing trends of GM adoption are included for reference and ates the popularity of the available varieties with farmers.

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### Transgenic Cotton in Australia

Cotton is currently the only transgenic crop variety in Australia licensed for commercial production. In the 2004/05 cotton season, some 80% of the 314,441 ha of cotton grown in Australian contained at least one genetic modification (*8*). There are two modifications currently available for cotton. Roundup Ready® varieties produce plants resistant to the herbicide glyphosate. Bollgard® II cotton incorporates two gene inserts (*Cry1Ac* and *Cry2Ab*) from *Bacillus thuringensis* (Bt), a soil borne bacteria, that produce proteins toxic to the cotton boll worm (*Helicoverpa sp.*) (*9*). These traits can be combined in the one cotton variety by classic plant breeding techniques to produce 'stacked' varieties that exhibit both traits (*10*). Stacked varieties accounted for approximately 50% of the genetically modified cotton grown during the 2004/05 season (*8*).

The main objective of these GM traits is to reduce the risk from pesticide use with cotton. For Roundup Ready® varieties, proponents claim that the use of glyphosate in conjunction with the GM variety will be more environmentally friendly that the use of more toxic residual herbicides on conventional varieties (*11*). In the case of Bollgard cotton varieties, the principal motivation for incorporation into integrated pest management regimes was a lower reliance on the insecticide endosulfan for *Helicoverpa* control with less risk of resistance. The adoption of these GM varieties is displayed in Figure 1.

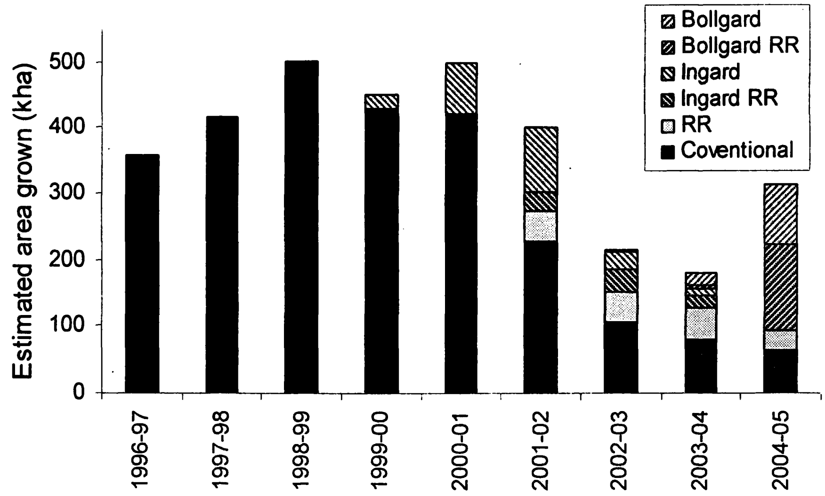

*Figure 1. Change in cotton varieties grown in Australia; adoption of GM technology as Bollgard® II, Ingard®, Roundup Ready® (RR) and stacked varieties. (Data from reference 8).*

## Hazard and Risk Reduction Assessment

A study was conducted to assess the environmental risk of glyphosate used for weed control on glyphosate-resistant genetically enhanced cotton varieties in relation to other commonly used herbicides, including diuron, 2,4-D [94-75-7], fluometuron [2164-17-2], pendimethalin [40487-42-1], prometryn [7287-19-6] and trifluralin.

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Risk assessments for a series of herbicide application scenarios were divided into two groups, GM and conventional, for comparison. The GM scenarios involved the cropping of genetically engineered, Roundup Ready® Sicot 189RR cotton, with glyphosate used as the main weed control chemical. Irrigation runoff data for glyphosate and other commonly used herbicides were obtained during field studies and used for a risk analysis in relation to water quality guidelines and the toxicology of two freshwater species, rainbow trout (*Oncorhynchus mykiss*) and water flea (*Daphnia sp.*).

The project consisted of a modelled risk assessment, which involved a series of theoretical herbicide application programs, and a field study involving herbicide treatment programs for weed control in cotton.

The field study was undertaken at Auscott Midkin in the Gwydir Valley (northern New South Wales, Australia) and consisted of four trial fields. All four fields were sown with Roundup Ready® Cotton (Sicot 189RR) to ensure no varietal differences were experienced. Two fields were subjected to typical conventional herbicide programs over the 2001/02 growing season whilst the other two were subjected to typical glyphosate-dominated herbicide programs. Other herbicides used in the study included 2,4-D, diquat dibromide [85-00-7], diuron, fluometuron, paraquat dichloride [1910-42-5], pendimethalin, prometryn and trifluralin. The herbicide application scenarios assessed were based upon industry-typical application programs over an entire growing season. The four fields, with gradients of 1:1400, consisted of light to medium grey clay soils. The soils were relatively uniform across the fields with clay content between 50-60%. Soil organic carbon levels were 1 ± 0.2%, and pH ranged from 7.5 to 8.6.

The results for runoff and suspended sediment (>45 μm) mirrored the dissipation rates observed in the soil samples. Concentrations of herbicides in runoff generally decreased during the season because of the decreasing field source. However, the total concentrations in runoff were affected by sediment load, which varied during the season. Glyphosate in runoff was only detected in suspended sediments, which were all well below ANZECC/ARMCANZ water quality guidelines (*12*). Conversely, diuron was observed to exceed the freshwater low reliability trigger value (0.2 μg $L^{-1}$) in every sample. Fluometuron and prometryn were applied to fields 29 and 84 only (i.e., conventional programs). Both herbicides were found to partition quite strongly into the aqueous phase. The field results are considered to be consistent with the understanding of the environmental fate and transport of herbicides (*13, 14, 15*).

## Risk of Exceeding Prescribed Water Quality Guidelines

From the runoff observations made during this study, the probability of exceeding the local guideline values was taken as a measure of risk to the aquatic

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environment (Table I). These probabilities were determined from an exponential line equation fitted to the frequency distributions of the residues detected in the runoff sampling. The values indicate the likelihood of exceeding the guideline values under normal agricultural practice, as experienced during the trial. No special experimental conditions were imposed during the study; all chemicals were applied by routine operators using typical application equipment.

**Table I: Probabilities that pesticide concentrations in runoff water from cotton fields will exceed the environmental guideline values**

| *Chemical* | *Guideline ($\mu g\ L^{-1}$)* | *Conventional (P>Guideline)* | *GM (P>Guideline)* |
|---|---|---|---|
| Diuron (ID[b]) | 0.2 | 0.80 | 1 |
| Trifluralin | 2.6 | 0.58 | 0.58 |
| Fluometuron (MRL[a]) | 100 | 0.08 | - |
| Prometryn (MRL[a]) | 100 | $1.0\times10^{-5}$ | - |
| Pendimethalin (MRL[a]) | 50 | $3.0\times10^{-6}$ | - |
| Glyphosate | 370 | $1.9\times10^{-10}$ | $8.8\times10^{-11}$ |

[a] Where no ANZECC/ARMCANZ (*12*) guideline values were prescribed MRL values were used.

[b] Refers to the ANZECC/ARMCANZ (*12*) "Insufficient Data" condition and therefore a value with greater sensitivity is prescribed.

The ANZECC/ARMCANZ water quality guidelines for environmental protection (*12*) do not list values for all herbicides registered for use in Australia. To enable some comparison between the products used in this study, surrogate 'maximum residue level' (MRL) values were used for fluometuron, prometryn and pendimethalin. Diuron was detected at concentrations greater than 0.2 $\mu g\ L^{-1}$ in every sample from the GM fields, with a resulting large probability of exceeding the guideline value. However, the guideline concentration (*12*) for diuron is a 'low reliability trigger value' and is conservative to ensure ecosystem protection because there is insufficient toxicity data available to determine a more realistic value.

Glyphosate posed the smallest probability of exceeding the prescribed guideline value (from Table I). This is likely a combined result of its relatively low mobility, reported in the literature and shown by the lack of detection in runoff samples (*16*) and its low toxicity and subsequent relatively large ecosystem protection guideline value.

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## Relative Risk to Aquatic Ecosystems for Field Experiment Scenarios

The field trial results were used to determine the probability of exposure and the average and median level of exposure. An expression of actual risk was difficult given the scale of the experiment, however a relative assessment including a factor based upon observations was produced. The probability of exposure was determined using observations above the limit of detection (LOD) in the runoff and suspended sediment samples. Exposure values (X) were determined by $X = C \times P \times t_{1/2} \times BCF$. Where C is the concentration in runoff, P is the probability that residues will be found in runoff, $t_{1/2}$ is the half-life, indicating persistence, and BCF refers to the bioconcentration factor.

The concentrations of herbicides used for the ecosystem risk assessment detailed in this section are shown in Table II. The average and median data were determined from the data set obtained from runoff in the field trials. The median values were selected to represent exposure values.

**Table II: Average and median values of the herbicide concentrations in runoff reported from the field trials**

| *Chemical* | *Conv. Ave. (μgL⁻¹)* | *GM Ave. (μgL⁻¹)* | *Conv. Median (μgL⁻¹)* | *GM Median (μgL⁻¹)* |
|---|---|---|---|---|
| Glyphosate | 5.33 | 8.46 | <10 | <10 |
| Diuron | 38.05 | 19.34 | 43.68 | 7 |
| Fluometuron | 25.56 | 1.10 | 11.9 | 0.9 |
| Prometryn | 4.34 | 1.04 | 2.09 | 1.2 |
| Pendimethalin | 2.61 | 0.36 | 1.91 | 0.18 |
| Trifluralin[a] | 1.23 | | 0.42 | |

[a] Combined conventional and GM as no significant difference was found between application programs

In the case of glyphosate a negative BCF was given, because of its high aqueous solubility and negative log $K_{OW}$. As such, the negative BCF was disregarded for the exposure calculations contained in Table III. Risk to fauna and flora in ecosystems was determined by estimating the toxicity of the herbicides used in the field scenarios to rainbow trout and water flea. The $LC_{50}$ toxicity values of the relevant chemicals for these species are listed in Table IV.

Applying the information on exposure and toxicity as described in Tables III and IV to the equation, $RR = X/Tox$, where X is the exposure and Tox is the toxicity, gives the relative risk (RR) to the ecosystem.

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**Table III: Determination of exposure of each chemical to the ecosystem**

| *Chemical* | *Probability (P) = n(+)/N* | | *Half-life ($t_{1/2}$)* | *BCF* | *X = C x P x $t_{1/2}$xBCF* | |
|---|---|---|---|---|---|---|
| | *Conv (P)* | *GM (P)* | *days* | *log* | *Conv.* | *GE* |
| Glyphosate | 0.33 | 0.46 | 22 | -2.59 | 0.07[a] | 0.10[a] |
| Diuron | 1.00 | 1.00 | 60 | 3.15 | 8256 | 1323 |
| Fluometuron | 1.00 | - | 85 | 2.73 | 2761 | - |
| Prometryn | 1.00 | - | 60 | 3.38 | 424 | - |
| Pendimethalin | 1.00 | - | 21 | 5.23 | 210 | - |
| Trifluralin[a] | 0.89 | | 25 | 4.92 | 45.6 | |

[a] Combined runoff data, conventional and GM

**Table IV: Input data on environmental fate and toxicity**

| *Chemical* | *$K_{oc}$*[a] | *$t_{1/2}$*[b] *(days)* | *Fish $LC_{50}$*[c] *($mgL^{-1}$)* | *Daphnia $LC_{50}$ ($mgL^{-1}$)* |
|---|---|---|---|---|
| 2,4-D | 100 | 10 | 100 | 0.24 |
| Clethodim | 200 | 90 | 2-56 | 25 |
| Diuron | 400 | 90 | 1.1-5.6 | 12 |
| Diquat | 100000 | 1000 | 21 | - |
| Fluometuron | 100 | 85 | 47-53.5 | 54 |
| Glyphosate | 24000 | 47 | 1000 | 780 |
| Metolachlor | 200 | 90 | 2 | 25 |
| Paraquat | 100000 | 1000 | 32 | - |
| Pendimethalin | 5000 | 90 | 0.14 | 0.28 |
| Prometryn | 400 | 90 | 2.5 | 12.7 |
| Pyrithiobac | - | - | 930[d] | 110 |
| Trifluralin | 8000 | 60 | 0.01 | 0.56 |

[a] Sorption co-efficient ($K_{oc}$) represents the binding affinity of a pesticide to organic matter and is an indicator of its mobility.

[b] Half-life represents the relative persistence of different pesticides in soil.

[c] $LC_{50}$ value corresponds to the concentration at which 50% test organisms mortality was observed (*17*).

[d] Toxicity for Bluegill (*17*).

Using the relative risk categories shown in Table V, results of the risk assessment are presented in Table VI. It should be noted that the assignment of relative risk categories given applies to the current study and should not be used out of this context.

**Table V: Relative risk to the ecosystem of the herbicides used in the field trial scenarios**

| | | *Relative Risk - Conventional Scenarios* | | |
|---|---|---|---|---|
| *Chemical* | *Fish* | *Category*[c] | *Daphnia* | *Category* |
| Glyphosate | $7.3\times10^{-8}$ | Negl. | $7.9\times10^{-8}$ | Negl. |
| Diuron | 7.5 | Med. | 0.2 | Low |
| Fluometuron | 0.05 | Low | 0.3 | Low |
| Prometryn | 0.08 | Low | 0.01 | Low |
| Pendimethalin | 4.2 | Med. | ID[b] | - |
| Trifluralin[a] | 1139.4 | High | 0.08 | Low |
| | | *Relative Risk - Genetically Modified Scenarios* | | |
| *Chemical* | *Fish* | *Category* | *Daphnia* | *Category* |
| Glyphosate | $1.0\times10^{-7}$ | Negl. | $1.1\times10^{-7}$ | Negl. |
| Diuron | 1.2 | Med. | 0.03 | Low |
| Fluometuron | - | - | - | - |
| Prometryn | - | - | - | - |
| Pendimethalin | - | - | - | - |
| Trifluralin[a] | 1139.4 | High | 0.08 | Low |

[a] Combined Conventional and GM data

[b] (ID) Insufficient data

[c] RR>10 (High or 3) 1<RR<10 (Medium or 2); 0.01<RR<1 (Low or 1); RR<0.01 (Negligible or 0).

Similar conclusions to Table III can be drawn from the data presented in Table V for glyphosate. Of all the herbicides, glyphosate generates the least risk of exceeding the criteria used – in this case toxicity to two aquatic species. Not surprisingly, the risk for diuron was smaller than that suggested by the previous assessment, which resulted from a highly protective guideline value (*12*). From the limited comparisons available, it appears that diuron poses a risk similar to pendimethalin; both chemicals are categorized to pose medium relative risk to trout. Of the chemicals examined, trifluralin posed the greatest relative risk to trout.

In assessing the conventional versus GM herbicide programs used in this trial, it can be concluded that the use of prometryn and fluometuron in a GM program instead of trifluralin and diuron would result in a reduction of risk associated with the overall GM program. However, such a reduction of risk would depend upon the interchangeability of the herbicide treatments. In order to maintain the low risks from use of fluometuron and prometryn, the current rates of application would need to be maintained, as an increase in application rate would increase exposure concentrations and associated risk to ecosystems. One

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of the main principles of ecosystem protection, from a chemical residue aspect, is that the lower the mass of chemical applied to the environment the lower the ecological risk from contamination.

**Table VI. Pesticide reductions by SSF channels. Asterisks indicate significant differences ($p<0.05$) relative to the performance of open channels.**

| | | *Irrigation 1* | *Irrigation 2* | *Irrigation 3* |
|---|---|---|---|---|
| Fluometuron | Inlet ($\mu gL^{-1}$) | 3.1 | 82 | 14.3 |
| | Outlet ($\mu gL^{-1}$) | 2.9 | 48.4 | 15.9 |
| | Reduction (%) | 7 | 41* | -10 |
| Endosulfan | Inlet ($\mu gL^{-1}$) | nd[a] | 2.05 | 0.5 |
| | Outlet ($\mu gL^{-1}$) | | 1.52 | 0.66 |
| | Reduction (%) | | 26* | -24* |
| Diuron | Inlet ($\mu gL^{-1}$) | 32.9 | 3 | nd |
| | Outlet ($\mu gL^{-1}$) | 29.4 | 2.4 | |
| | Reduction (%) | 11 | 20 | |
| Prometryn | Inlet ($\mu gL^{-1}$) | nd | nd | 2.1 |
| | Outlet ($\mu gL^{-1}$) | | | 1 |
| | Reduction (%) | | | 52* |

[a] below limit of detection

## Risk to Aquatic Ecosystems for the Theoretical Herbicide Application Scenarios

It was not possible to include all herbicides in the field trial because of limited resources. The fugacity approach (*18, 19*) was used to approximate herbicide concentrations in runoff from the theoretical herbicide scenarios, including 2-4 D, which is used in cotton production for field preparation, although not on cotton because of phytotoxicity. These data were used to determine a hazard quotient based upon the toxicity data presented in Table IV. The results were then given a score based upon the magnitude of the resultant quotient (see Table V). The hazard quotient scores for the two ecological species were then averaged and computed for each of the 10 theoretical application scenarios.

The results from the theoretical scenarios showed good agreement with the risk categories determined for the actual field experiment scenarios. Diuron and trifluralin consistently gave high risk to trout and low and medium risk respectively to daphnia. The assessment of fluometuron indicated a high and

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medium risk for *Daphnia* and trout respectively. These results differed from the assessment using the MRL guideline value and are considered to be a better characterization of potential risk. The theoretical scenarios allowed metolachlor, pyrithiobac-sodium and clethodim to be assessed and these results showed that the risk of using these chemicals at the indicated rates was usually negligible. The risk associated with the use of glyphosate was consistently found to be negligible.

In general, a low risk categorization for a particular herbicide resulted from having a low surface water mobility and low toxicity to the species studied. Scores were assigned to each risk category for the theoretical herbicide scenarios and these scores were used to compare the herbicide scenarios. It was found that the GM programs did not offer a significantly lower combined score. From a practical or management viewpoint, the scoring system could be used to choose a herbicide application program consisting of the herbicides that return a low relative risk score and hence pose the least risk to aquatic ecosystems.

Figure 2 shows a comparison of all herbicides used in the theoretical scenarios, with the risk scores from each scenario averaged to provide a comparison of each herbicide treatment.

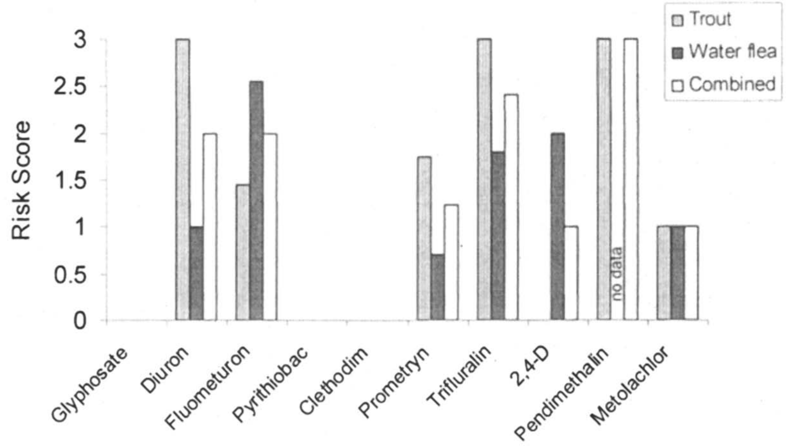

*Figure 2. Risk score for each herbicides, presented as averages of all scores from all scenarios*

Some herbicides, such as glyphosate, prometryn and fluometuron, were routinely found to present 'negligible' or 'low risk' of either exceeding water quality guidelines or threatening the ecosystem species assessed. This reinforces the suggestion that certain chemicals, such as glyphosate, metolachlor, 2,4-D, pyrithobac-sodium and clethodim, could be included in a 'low risk' herbicide program.

Whilst modelled risk assessments can be used as an indication of likely risks, the field data and associated risk assessment provides a superior basis for management decisions. The model approach is useful for identifying which herbicides could be the focus of field trials to obtain better environmental information concerning both the environmental fate of herbicides and the toxicity of herbicides to local ecosystems, including plant species.

### The Changing Spectrum of Pesticide Use

Contemporaneously with the cotton industry's drive to foster populations of cotton-pest predators within cotton crops (*20*), cotton growers have been educated to reduce the number of applications of broad-spectrum insecticides, and, when possible, total insecticide applications.

Figure 3 shows the variability of total insecticide use from season to season, depending primarily on pest pressure, likely a function of pre-season rainfall. However, there is no clear evidence that the total mass of insecticide applied per hectare of conventional crop has been significantly reduced over the last 10 years. Nevertheless it can be seen that the total mass of insecticide applied to GM cotton is significantly less that that on conventional, which, taken with the increasing area GM crop grown, indicates an overall reduction in the mass of insecticide used across the industry.

Further analysis of this data on changing insecticide use would allow risk assessment to be performed comparing the conventrional cotton with GM cotton. This work is progress, however, it is obvious that reductions in pesticide use with Bollgard II represents a reduction in risk.

More specifically, records indicate a shift in the type of pesticides being used (Figure 4). It can be seen that there has been a decrease in the use of pesticides classified as 'broad-spectrum' from an average of 14 sprays per crop in 1996/97 to an average of less than four sprays per crop in 2001/02. There has been a simultaneous increase in the use of more 'selective' pesticides.

## Artificial Wetlands for Risk Reduction

Of the environmental compartments into which pesticides can partition, contamination of fresh water sources often presents the highest risk because of its ubiquitous use by organisms and high potential for direct exposure (*13, 21*). One best management practice gaining current attention is the use of constructed wetlands to treat pesticide-contaminated runoff water at the source. The usefulness of this technology arises from it being relatively inexpensive, robust and possible to integrate into current land management practices.

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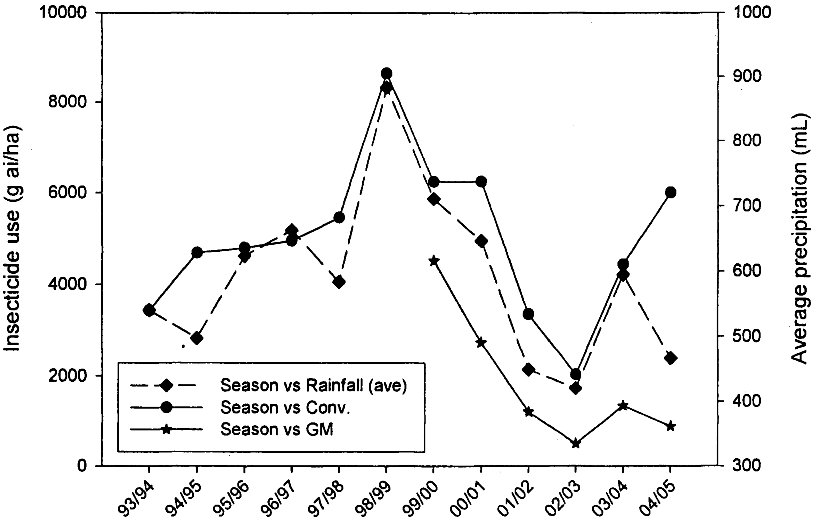

*Figure 3. Twelve season record of total insecticide use (per ha) of conventional (Conv.) and genetically modified (GM) cotton. (Data from reference 8)*

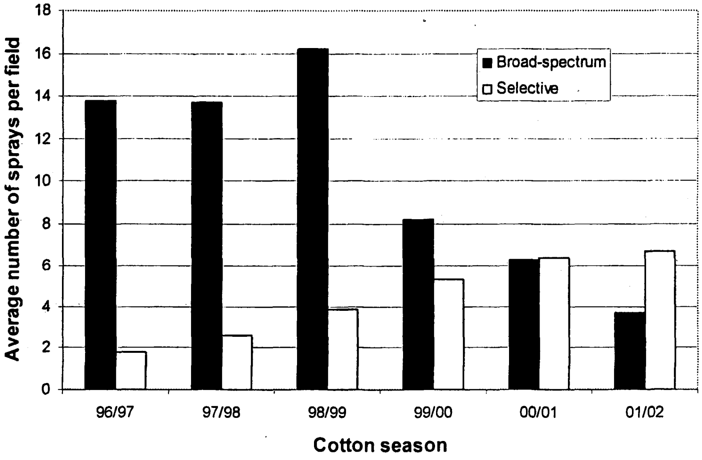

*Figure 4. Changing pattern of use of pesticides in the Australian Cotton Industry (Data from reference 8).*

Evidence from studies on constructed wetlands for this purpose indicates that aquatic vegetation can accelerate pesticide removal compared to open water systems. The main reason reported for this in the literature is the increased opportunity for plant/biofilm sorption and subsequent immobilization, breakdown or uptake of water-borne pesticides (*22*).

Two complementary methods investigated in the Australian cotton industry were the use of sub-surface flow wetlands, for immediate sorption and filtration of pesticides from irrigation tailwater at the field exit, and surface flow wetlands in tail-drains and storage dams to accelerate passive breakdown of residues. It was anticipated that these techniques would reduce acute and chronic risk, respectively, to wildlife using on-farm tailwater as a food and habitat source.

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## Sub-Surface Flow Pilot-Scale Wetland

Two sub-surface flow (SSF) tail-drains were constructed adjacent to two open, control tail-drains on a cotton property in the Naomi Valley, NSW, Australia. The SSF drains contained 7 mm basalt gravel as a filter medium and were of dimensions 20 m length, 2 m width and 0.5 m depth, consisting of four baffled compartments. Open drains were the same design without gravel media. Inlet and outlet concentrations of pesticides in diverted tailwater were measured during three irrigation events over one cotton season.

Results showed that the SSF drains could reduce and spread out peak concentrations of pesticides. However, washoff of previously sorbed pesticide (fluometuron and endosulfan) was observed during treatment of tailwater in late season irrigations with smaller inlet pesticide concentrations (Table VI), demonstrating the persistence of sorbed residues.

Thus, SSF tail-drains are best used to avoid acute risks involved high spike concentrations of pesticide contaminated runoff. Work is currently investigating the potential for using recycled cotton ginning waste and crop residues as a filter medium, with high organic matter media showing greater binding capacity than inorganic media.

## Ponded Wetlands

A pilot-scale ponded wetland, consisting of an non-vegetated and a vegetated pond in series, was constructed and assessed for pesticide dissipation over three cotton growing seasons. Tailwater was diverted from a cotton field treated with various pesticides and held for 10-12 days, with pesticide concentrations being measured at the start and end of the holding period.

Results over the three years of study were variable. In the first year of study, aquatic plant growth was not rapid enough to bring about a difference in the pesticide dissipation rates between the vegetated and non-vegetated ponds, hence average half-lives were calculated (Table VII) (*23*). In the second and third seasons, pesticide dissipation was significantly more rapid across the entire ponded system (both vegetated and non-vegetated ponds), with the vegetated pond generally exhibited more rapid pesticide dissipation. However, under certain circumstances rates of dissipation were faster in the open pond, mainly due to algal growth, and the reader is referred to Rose *et al.* (*23*) for further information.

Increased dissipation of pesticides in constructed wetlands prior to release to other surface waters could be used as a basis for estimates of relative risk for various chemicals, taking probability of exposure into account as demonstrated previously in this chapter.

**Table VII. Half-lives (days) of pesticides in tailwater treated by the pilot-scale ponded wetland. (95% confidence limits in parentheses).**

| | | *Season 1* | *Season 2* | *Season 3* |
|---|---|---|---|---|
| Aldicarb | Open pond | 26 (7) | 6 (0.3) | nd[a] |
| | Vegetated pond | | 5 (0.3) | |
| Endosulfan | Open pond | nd | 7 (0.2) | 5 (0.6) |
| | Vegetated pond | | 6 (0.5) | 4 (0.7) |
| Diuron | Open pond | 21 (4) | nd | nd |
| | Vegetated pond | | | |
| Fluometuron | Open pond | 25 (9) | 14 (1) | 31 (9) |
| | Vegetated pond | | 10 (1) | 12 (4) |

[a] below limit of detection

## Conclusion

The introduction of GM cotton varieties has influenced pesticide use in the Australian cotton industry. It is clear that pesticide use has been reduced, and that GM cotton varieties enable the use of lower risk herbicides or more selective insecticides. The result has been a reduction of environmental risks, as indicated in Table VIII by a decreasing number of detections of pesticides in rivers adjacent to cotton production (*24-26*). However, pesticides remain an important input for cotton production, and hence other risk mitigation options need to be considered.

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**Table VIII: Thirteen year record of the number and percentage of detections of common pesticides in the Namoi, Gwydir and Macintyre Rivers.**

| *Year* | *Endo*[a] | *Atra* | *Diur* | *Fluo* | *Meto* | *Prom* | *Sima* | *No.* |
|---|---|---|---|---|---|---|---|---|
| 1991/92 | 174<br>(59%) | 136<br>(46%) | 60<br>(20%) | 16<br>(5.4%) | 0 | 41<br>(14%) | ns[b] | 296 |
| 1992/93 | 194<br>(65%) | 113<br>(38%) | 28<br>(9.4%) | 17<br>(5.7%) | 0 | 32<br>(11%) | ns | 299 |
| 1993/94 | 137<br>(65%) | 71<br>(34%) | 28<br>(13%) | 19<br>(9.0%) | 14<br>(6.7%) | 15<br>(7.1%) | ns | 210 |
| 1994/95 | 135<br>(48%) | 106<br>(38%) | 27<br>(9.6%) | 10<br>(3.6%) | 2<br>(0.7%) | 12<br>(4.3%) | ns | 281 |
| 1995/96 | 169<br>(58%) | 178<br>(61%) | 14<br>(4.8%) | 2<br>(0.7%) | 25<br>(8.6%) | 23<br>(7.9%) | 0 | 291 |
| 1996/97 | 207<br>(52%) | 138<br>(35%) | 24<br>(6.0%) | 32<br>(8.1%) | 21<br>(5.3%) | 39<br>(9.9%) | 0 | 395 |
| 1997/98 | 196<br>(49%) | 86<br>(21%) | 40<br>(10%) | 70<br>(17%) | 37<br>(9.2%) | 48<br>(12%) | 3<br>(0.7%) | 404 |
| 1998/99 | 182<br>(46%) | 131<br>(33%) | 79<br>(20%) | 73<br>(18%) | 53<br>(13%) | 31<br>(7.8%) | 8 (2%) | 400 |
| 1999/00 | 126<br>(31%) | 177<br>(43%) | 75<br>(18%) | 66<br>(16%) | 58<br>(14%) | 35<br>(8.5%) | 2<br>(0.5%) | 413 |
| 2000/01 | 76<br>(17%) | 184<br>(42%) | 57<br>(13%) | 86<br>(20%) | 59<br>(14%) | 25<br>(5.7%) | 18<br>(4.1%) | 438 |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2001/02 | 14<br>(4.8%) | 81<br>(28%) | 28<br>(9.7%) | 21<br>(7.2%) | 15<br>(5.2%) | 17<br>(5.9%) | 18<br>(6.2%) | 290 |
| 2002/03 | 4<br>(1.1%) | 69<br>(20%) | 27<br>(7.8%) | 18<br>(5.2%) | 9<br>(2.3%) | 10<br>(2.9%) | 3<br>(0.8%) | 348 |
| 2003/04 | 1<br>(0.3%) | 128<br>(37%) | 29<br>(8.4%) | 19<br>(5.5%) | 17<br>(4.9%) | 12<br>(3.5%) | 5<br>(1.5%) | 344 |

[a] Columns are, from left to right: endosulfan, atrazine, diuron, fluometuron, metolachlor, prometryn, simazine, number of samples analysed.

[b] ns = not sampled

SOURCE: Data from references 24, 25 and 26.

We have presented data on the use of wetlands, as a buffer within irrigated cropping systems, to afford greater protection of natural ecosystems.

The apparent trend between pesticide use and rainfall, as a precursor of pest pressure, (Figure 3) deserves more analysis and will be the focus of further work. This would enable a better understanding of pesticide use data and biotechnology benefits, as well as facilitating accurate comparisons between seasons and regions. There is little doubt that the use of GM cotton varieties reduces pesticide use and associated environmental risk. The extent of risk reduction should be referenced against climatic conditions.

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## Chapter 21

# Lessons for Achieving Effective Management from Field Research on Agrochemicals

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Australian cotton and sugarcane growers have been the focus of increased community concern regarding the potential off-site impacts of pesticides on surface and ground waters. For the cotton industry, the off-site losses to riverine systems of the insecticide endosulfan was a major issue. For the sugar industry, the herbicides atrazine and diuron were the most widely used, with environmental concern linked to possible contamination of both surface and groundwaters, heightened by the proximity of the Great Barrier Reef. In response, over the past decade, multidisciplinary field-based research sites were established in tropical and sub-tropical Queensland as well as in tropical Mauritius. Together with detailed studies on field persistence and mobility of the pesticides, sites were established with devices to quantify off-site losses via runoff, leaching or preferential flow pathways. Site-specific studies are essential to provide confidence and credibility in the development of science-based management guidelines. Scientific findings need to be presented in a concise format that maximise information transfer and acceptance. These issues are presented here.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

# Introduction

With annual production areas of approximately 550,000 and 400,000 hectares respectively, Australia's cotton and sugar production uses a range of agricultural chemicals to optimise productivity. Their use in close proximity to waterways and urban development has sometimes resulted in media pressure calling for the termination of pesticide use or farming in the area. Community concerns include both the human and environmental health issues, often triggered by off-farm spray drift. Some spray drift cases have also been associated with serious economic damage, resulting from direct (e.g. herbicide damage) or non-direct (e.g. cattle grazing on contaminated pastures or damage to pollen producing plants affecting honey production) mechanisms. Australia has some existing regulatory systems (*1-3*) in place, which help to minimise such cases.

Most environmental concerns have related to potential contamination of water resources (*4*) and the impact this may have on the ecosystem. There are several off-site transport mechanisms such as spray drift, volatilisation, dust migration, surface runoff and leaching, but farm runoff or leaching processes trigger most environmental concerns. Unfortunately, there are limited monitoring data on pesticide residues in surface and groundwaters in Australia. The limited data tend to be associated with short-term projects rather than long-term state or national monitoring studies. However, the data available (*5*) indicate that some pesticide residues are entering the river systems, usually at higher rates during the pesticide application season.

For the cotton industry, the off-site loss of the insecticide endosulfan was a major issue, particularly the frequency of endosulfan residues (*5*) being detected in inland river systems. This situation triggered a major national research program entitled 'Minimising the Impact of Pesticides on the Riverine Environment – Using the Cotton Industry as a Model'. For the sugar industry, environmental concerns, particularly regarding the widespread use of the herbicides atrazine and diuron, were linked to the close proximity of the Great Barrier Reef, downstream from the sugar production areas of Queensland (*6*, *7*).

Both the Australian cotton and sugarcane industries have developed industry guidelines (*8-11*) for best practice and have programs in place for continual improvement. Such guidelines cover the overall management of the farming system and include components designed to minimise environmental impacts by their industry. Most cotton and sugarcane growers believe they are environmentally responsible regarding pesticide application and are receptive to change providing it is practical and based on sound reasoning rather than some artificially imposed constraint. However, with limited information available to growers on the in-field fate and persistence of pesticides used and little knowledge available on the off-site transport processes, these industries were

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vulnerable to such community and regulatory pressures which could threaten their economic viability if key pesticides were prematurely withdrawn. Whilst excellent literature data are available for most pesticides (*12*), there is often limited information on their behaviour within operating farming systems, particularly under tropical conditions. Because the dissipation and mobility of pesticides can be affected by variables such as soil type, temperature, site drainage and cultural practices, some site-specific studies were considered essential to provide credible management guidelines.

To address this issue, multidisciplinary field research sites were established over the past decade in tropical and sub-tropical Queensland as well as in the high-rainfall area of tropical Mauritius. With extensive sugar production areas (70,000 hectares), Mauritius had similar concerns to Australia regarding potential contamination of water supplies or their surrounding reef system and sought help from Australia to undertake appropriate research. Together with detailed studies on field persistence and movement of the pesticides, field sites in cotton and sugar production areas were established with flumes to quantify runoff and with moisture probes, piezometers or related devices to assess leaching or preferential flow pathways. Such high-cost studies were made possible by multiple funding sources from both industry and government research organisations, with the individual projects in tropical areas being led by the Queensland Department of Natural Resources and Mines, a State Government Department. The overall investment in this tropical component of the research over the 10-year period was approximately A$4.8 million (US$3.6 million).

An overview of the major field studies and some comment on their outcomes are discussed, together with some suggestions on improving the delivery and utility of such field-based research, not only to the production industries involved but also to the broader community including research organisations, policy makers and the general public.

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## Research Methodology

### A. Field Studies for Cotton

A field site was established in the Emerald Irrigation Area (EIA) of Central Queensland (S23$^0$30', E148$^0$07') where constant high summer temperatures and favourable conditions during the growing season resulted in high insect pressure, particularly from *Heliothis*. Average annual rainfall in the area is approximately 620mm, mainly from summer storms. Regular applications of endosulfan in the earlier part of the season had proven to be highly effective in controlling

*Heliothis*. Normal application rates were 3L/ha ULV [240g/L] (aerially applied), with up to 10 applications per year when high insect pressure existed. These applications combined with the use of other insecticides and herbicides, increased the concerns regarding environmental contamination.

The typical brown cracking clay site was equipped with instruments (Figure 1) at plot scale (6 rows x 250m), on two blocks with flumes, bed-load traps, runoff height recorders, loggers and automatic samplers. Taildrain outlets from the two blocks (approx. 80 hectares each) were fitted with weirs, height recorders, loggers and automatic samplers (containing 1L glass bottles). Detailed recordings were kept of farm practices, crop growth, pesticide inputs, meteorological data (collected on farm) and irrigation details.

Samples of soil, sediment and runoff were taken throughout the crop cycle and analysed for pesticide residues to determine both the persistence of the pesticide on-farm (potential for runoff) and the concentration in the runoff. Hydrological data, collected for all runoff events, were used to calculate the total load of pesticide leaving the farm. The effect of cotton stubble retention and added wheat straw on pesticide movement was studied in the second season. In the third season, an additional site was added to study the effect of a more extreme stubble treatment (pre-cotton wheat crop) on pesticide retention.

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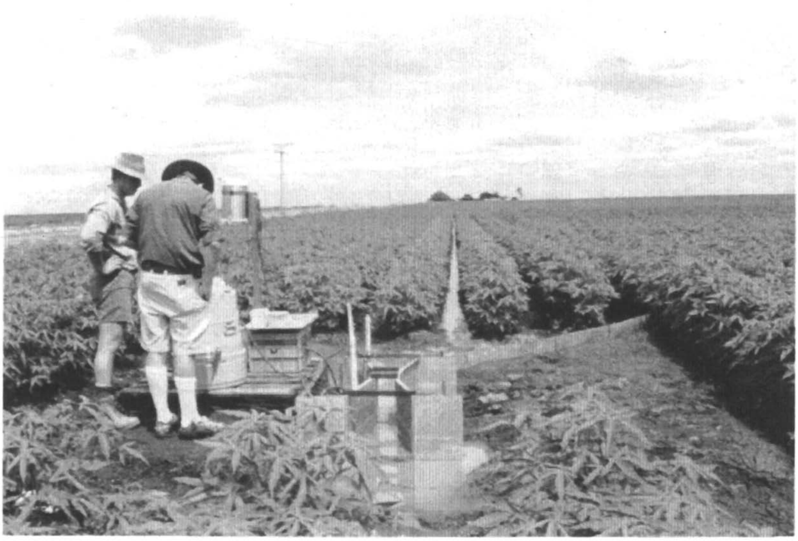

*Figure 1. Instrumentation installed at plot-scale in central Queensland for measuring and sampling runoff*

## Key Results and Discussion on Cotton Studies

### *Pesticide Residues in Soils*

In the high-clay (70-80% clay) soils of EIA, there was no evidence of build-up of pesticide residues in the soil from season to season - either in the surface layer (0-2.5 cm) or down the profile (approx. 1m). Compounds measured included endosulfan (α, β and sulphate), trifluralin, prometryn, fluometuron, diuron and methyl parathion. DDE, the persistent breakdown product of the previously used insecticide DDT, was also detected in all samples analysed.

The herbicide trifluralin, which was applied pre-planting (incorporated to 10 cm) was found to be relatively persistent in the soil and slowly declined throughout the season. Prometryn and fluometuron, applied to hills (plant rows) at planting, declined moderately (dissipation rate [$DT_{50}$] of approximately one month) and diuron, applied to furrows at last cultivation, declined rapidly, particularly when furrow irrigation (or runoff from rain) was applied immediately after application

Following the first endosulfan application, residues in the surface soil (0-2.5cm) rose to approximately 2 mg/kg (application of 3 L/ha ULV aerially applied) and showed reasonably rapid decay (dissipation rate of approximately one week for total endosulfan), a process where both the original α and β isomers are reduced by a number of mechanisms, including conversion to endosulfan sulfate. Multiple applications of endosulfan (up to 10 per season in the EIA in 1993-94) resulted in slow build-up (because of on-going breakdown) to approximately 4 mg/kg in the surface (0-2.5cm) layer (Figure 2).

This information on pesticide levels in the soil, clearly shows that the major potential risk for off-site movement of these pesticides from the cotton field is limited to the early part of the season when soil residues are the highest. The highest risk periods for the more mobile herbicides eg prometryn, fluometuron and diuron are more confined to the period 4-6 weeks after application.

While endosulfan loading on the soil was reduced by approximately half for each week after application, repeated applications of endosulfan resulted in a short-term build-up of residues. This extended the risk period for endosulfan losses and helped to explain the detection of endosulfan in runoff waters over a large part of the cotton season.

### *Off-farm Movement of Pesticides*

Pesticide concentrations leaving the end of furrow or taildrain were relatively high, typically 8-15μg/L for endosulfan (approximately 1000 times the then environmental guideline (*13*) of 0.01μg/L in rivers) during the peak of the

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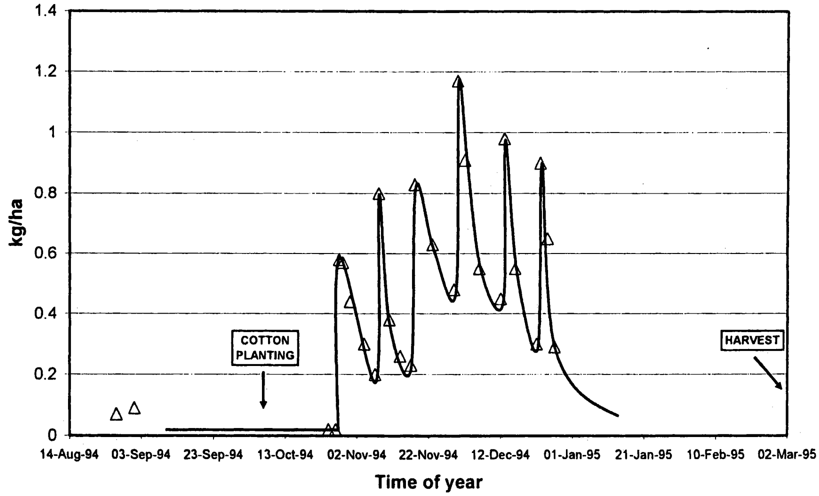

*Figure 2. Total endosulfan in soil from six aerial applications during cotton growing season*

season for irrigation runoff, dropping to approximately 2-3μg/L towards the end of the season. Similar levels were found in the main drainage channels of the EIA throughout the season. Much higher levels could be expected for major storm runoff in peak season. Herbicides such as prometryn, fluometuron and diuron, which were applied once in a season, produced relatively high concentration (e.g. 40μg/L for diuron) in irrigation runoff, particularly in the immediate weeks following application.

Hydrological measurements combined with suspended sediment, bedload and pesticide data helped to quantify the transport processes. The amount of pesticide leaving the taildrain after a typical furrow irrigation was calculated to be in the order of 0.5 to 1.5% of the pesticide present in the soil at the time – highlighting the important link between soil residues and off-site losses.

Laboratory studies on soil/water partition coefficients (Kd) for the major pesticides enhanced the predictability of the movement pathways and thus provided an ability to quantify the link between management practices and off-site movement. These laboratory studies (Table I) showed that, in a soil/water mix, up to 70% of endosulfan (α, β, and sulphate) would partition onto the soil – giving a clear indication of the potential impact of effective sediment retention.

Field trials comparing three surface treatments [rake and burn (existing practice); cotton stubble retention; and wheat straw added (2-3 tonne/ha)] showed no significant effect on soil loss, bedload or suspended sediment and thus pesticide loss. The cotton stubble and added straw treatments may have been effective in reducing pesticide losses for an initial rainfall runoff event (had

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**Table I. Effect of sediment concentration on pesticide adsorption**

| *% sediment*[##] | *Trifluralin* | | *Prometryn* | | *α–endosulfan* | | *β-endosulfan* | | *E. sulphate* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *%** | *Kd*[#] | *%** | *Kd*[#] | *%** | *Kd*[#] | *%** | *Kd*[#] | *%** | *Kd*[#] |
| 0.1 | 55 | 1220 | 7 | 177 | 51 | 1070 | 56 | 1300 | 44 | 795 |
| 0.5 | 67 | 409 | 6 | 13 | 60 | 299 | 62 | 326 | 46 | 168 |
| 1.0 | 72 | 254 | 14 | 17 | 67 | 197 | 73 | 267 | 53 | 125 |
| 2.0 | 83 | 255 | 13 | 8 | 76 | 158 | 83 | 246 | 66 | 97 |
| 4.0 | 94 | 416 | 25 | 7 | 88 | 175 | 90 | 226 | 80 | 103 |

* Percent of pesticide adsorbed to soil phase

[#] Determined after 30 min equilibration (2 days after pesticide application)

[##] Brown cracking clay (70% clay in soil)

there been one resulting in runoff) but there was visual evidence that the stubble was quick to break down, thus any potential effect would be short-lived. Because of little rainfall, most significant runoff events during this trial season were from irrigation. Higher furrow slopes produced higher bedload and suspended sediment for all three plot-scale treatments.

Further treatments comparing conventional tillage, reduced tillage and pre-cotton wheat planting (wheat plants killed with glyphosate prior to cotton being planted directly into wheat stubble) demonstrated that the wheat stubble significantly reduced bedload and suspended sediment, particularly in the early to mid-season. For a heavy irrigation, only three days after the first endosulfan application (2.1L/ha), the endosulfan loss in the wheat treatment was only 27% of that from conventional tillage (Table II). Such reduction, predicted from the soil/water partition studies (Table I), was further confirmed in additional field studies in subsequent years.

## Summary of Key Findings and Related Guidelines:

- Cotton pesticides were not building up is the soil profile from season to season.
- Little evidence of pesticide leaching in the high-clay soils.
- Off-farm runoff of pesticides was mainly confined to the cotton-growing season – particularly following the pesticide application period.

**Table II. Effect of management change on runoff, sediment and endosulfan**

| *Treatment* | *% Reduction compared with conventional* management* | | |
|---|---|---|---|
| | *Suspended sediment in runoff* | *Total runoff* | *Total endosulfan in runoff* |
| Reduced Tillage | 32 | 22 | 10 |
| Pre-cotton wheat crop (sprayed out) | 73 | 61 | 73 |

* Cotton stubble raked and burnt followed by cultivation

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- Endosulfan loading on the soil (and thus potential for runoff) reduced by approximately half for each week after application.
- Repeated and frequent application of endosulfan increased endosulfan load (depending on application frequency) and thus enhanced the risk of off-site loss.
- For the herbicides prometryn, fluometuron, and diuron, the highest risk for offsite losses was in the period one month after application.
- The practice of applying diuron to furrows (just before canopy closure) and immediately furrow irrigating generated significant loss of diuron via runoff.
- Improved sediment retention (e.g. by maintaining solid stubble cover) could reduce endosulfan losses by 70%.
- Fewer endosulfan applications would be required reduce endosulfan load and thus further reduce risk periods.
- Improved water management (recycling, storm-water retention etc) would be needed to capture the more mobile herbicides and to further minimise off-farm losses of endosulfan.
- Where runoff retention was not practical, there would need to be increased attention given to risk periods, particularly in relation to potential storms and heavy rainfall.

## Cotton Industry Response to Research Findings

These field studies formed part of a national program on 'Minimising the Impact of Pesticides on the Riverine Environment – using the Cotton Industry as a Model', the research findings were given multiple exposure at a range of forums. Key industry leaders participated and were kept informed as new findings were presented and discussed, resulting in ownership at organisational level; research staff were able to deliver findings in a climate of constructive review, with knowledge that they were being supported by industry.

The simultaneous development of the cotton industry 'Best Practice Manual' allowed incorporation of research findings where appropriate, so that key lessons could be delivered to the broader cotton growing community. However, there were some missed opportunities in bringing research findings directly to growers. Since many Australian cotton growers use private advisors or consultants to help in their management operations, there was not a clearly identifiable extension group through which to process research findings or discuss management implications and options, particularly at local or regional level.

Growers in the Emerald Irrigation Area were influenced by the findings, and introduced strategies to reduce sediment losses, increased tailwater recycling and managed with fewer pesticide applications. The message that it is possible to significantly reduce off-site losses of pesticides without compromising productivity seemed to be broadly accepted, particularly where the research was being undertaken in the area.

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## B. Field Studies for Sugar

With the main sugar growing area of Australia extending over 2000km of the eastern coastline (Figure 3), site selection was designed to give some variability in growing conditions. The main study sites were established in subtropical (approx. 1000mm/year) Bundaberg area (S25$^0$06', E152$^0$18') of SE-Queensland, the dry tropics (approx. 780mm/year) area of Atherton Tableland (S17$^0$06', E145$^0$16') in North Queensland and in the wet tropical (>4000mm/year) Valetta area (S20$^0$16', E57$^0$33') of Mauritius. Both of the study areas in Queensland were supplemented by irrigation whereas the Mauritian sites were non-irrigated, similar to many of the wet tropical sugar production areas of Queensland.

A similar approach to that with cotton was used, with sites instrumented with flumes or weirs (Figure 4) to measure runoff as well as the addition of troughs, piezometers and moisture probes to examine sub-surface drainage. All studies were carried out under 'normal' farming practices on commercial farms, with full cooperation of the landowner. However 'normal' farm practice was augmented by the installation of hydrological, meteorological and sampling equipment, located to minimise impact on normal operations. Details were kept on all farming operations as well as irrigation and rainfall events. Most hydrological data collection was automated and logged so that useful time-series data were collected. Such data were useful when interpreting runoff from rainfall events and for calculating export loads of sediments and pesticides.

Surface and sub-surface soil samples (0-2.5, 2.5-5, 5-10, 10-20, 20-30 and 30-50 cm) were taken immediately before spay applications and for regular intervals following each application to determine dissipation rates ($DT_{50}$). It

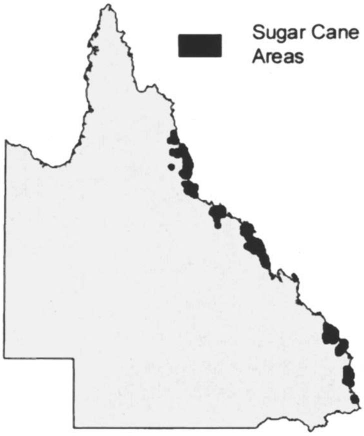

*Figure 3. Sugar growing areas of Queensland*

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should be noted that the field soils were subject to the normal farming practices of irrigation, cultivation and rainfall etc., with the soil profile subjected to some disturbance. Despite this, the data collected was statistically sound and provided 'real life' scenario information, rather than 'controlled' laboratory of glasshouse-generated data. First order kinetics were used to determine $DT_{50}$ and associated risk periods.

Soil water partition coefficients (Kd) were also determined for a number of the key pesticides on different of soil types at various time intervals after pesticide application. The purpose of undertaking these partition studies was to develop an improved understanding of the variability in soil/sediment adsorption by these pesticides and how this would contribute to the off-site loss potential via runoff or leaching.

## Key Results and Discussion on Sugar Studies

### *Pesticide residues in soils*

For atrazine, the most widely used herbicide used in Australian sugar production, $DT_{50}$s at the Bundaberg (SE-Queensland) were significantly faster than expected in the surface soil (0-2.5 cm), ranging from 1.0 day to a maximum of 27.5 days (the longest under very dry spring conditions) across the varied seasonal conditions, soil types and farming practices.

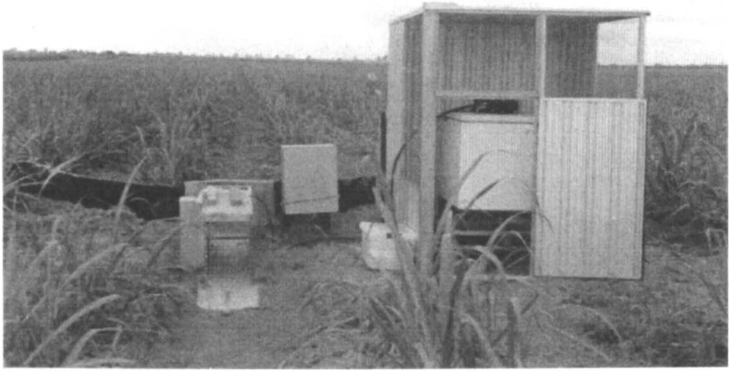

*Figure 4. Instrumentation at plot-scale (SE Queensland) for measuring and sampling runoff*

Apart from the one dry spring application, all other applications of atrazine were carried out in summer, between late October and late January where temperatures were higher and some rainfall or irrigation was experienced. The median dissipation rates in the 0-2.5 cm and 0-50 cm layers were 3.5 and 6.5 days respectively. Atrazine was rarely found below the 30 cm layer indicating that there was no evidence of sub-surface 'build up' in the soil profile.

The relatively low rainfall in the Bundaberg area compared with some other canegrowing areas (e.g. wet tropics), would suggest that the dissipation rates could be even faster in these wetter areas. However in the Mauritian studies, the maximum $DT_{50}$ measured in the surface (0-2.5cm) soil was 12 days – at a time prior to the commencement of the wet season. On these well-drained krasnozem soils, high rainfall could mobilise atrazine via leaching and preferential flow pathways. In such environments, atrazine should not be applied when heavy rains are expected, so that sufficient breakdown can occur before such rains or heavy irrigation commences. However, the findings from this project indicate that the time for dissipation under these tropical conditions is far shorter than previously thought, providing greater scope for scheduling atrazine application relative to expected rain.

The other most widely used herbicide in Australian sugar production is the phenylurea diuron, having different chemical and physical properties to atrazine (a triazine herbicide). Diuron is classed as relatively persistent and in this study was shown to have considerable variation in persistence, with $DT_{50}$ ranging from

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6.5 days to more than 250 days. As with atrazine, diuron was mainly confined to the top 30 cm, with little evidence of significant movement below this in the soil matrix. The mean $DT_{50}$ for diuron in the 0-2.5 cm layer (excluding a red ferrosol site) was 12 days. Dissipation curves for different pesticides under a range of field conditions were used to highlight risk periods (potential for pesticides to move off-site). Figure 5 shows such risk periods on the redoxic hydrosol site during the 1998-99 seasons.

The greatest contrast was found on one of the study sites, a red ferrosol (krasnozem) – also in SE-Queensland. On this site, diuron had not been previously used, and pre-application soil sampling showed no diuron residues present. Sampling of the soil (surface and sub-surface) from immediately after application showed that there was some initial dissipation and downward movement (to 30 cm), but afterwards, the remaining diuron was highly persistent ($DT_{50}$ >200 days) and 'immobile'. Such a 'two-phase' dissipation suggested that after the initial period of 'higher mobility', diuron was then adsorbed to, and protected within the soil matrix.

Soil/water partition coefficients (Kd) were determined on the four key soil types in the study area. All soils had typically low organic carbon, generally less than 1% in the surface layer and lower at depth. The findings (Table III) indicated considerable variability in (Kd), with reduced pesticide adsorption at depth. It should be noted that these data were generated using a 30 minute soil/water equilibration (20 g/L), two days after pesticide application.

To measure the effect of 'field ageing' of pesticide residues on soil adsorption, further laboratory studies were undertaken, with Kd values being determined at intervals over a 0-56 days. Data (Table IV) show considerable increase in soil adsorption following pesticide application, until a period of stability (in adsorption) is reached. This increased adsorption, combined with the normal pesticide degradation processes, provided further evidence for the benefits of effective soil/sediment management for minimising off-site losses.

### *Off-farm Movement of Pesticides*

Concentrations of pesticides measured in the runoff from the end of cane rows were variable and dependent on the pesticide levels in the soil at the time of the runoff event. Figure 6 shows the pesticide concentrations in the soil surface (0-2.5 cm) before and after a rainfall and runoff event which occurred 7 days after pesticide application. In this major runoff event (45 mm rainfall – 38% runoff), concentrations of atrazine, 2,4-D and chlorpyrifos in the runoff were 112, 6.8 and 21 μg/L respectively at the end of the cane row.

Based on the dissipation rates of the pesticides measured in the soil surface (0-2.5 cm), a similar runoff event seven days before (shortly after application)

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**Table III. Soil/water partition coefficients on selected sugarcane soils**

| *Site* | | *Yellow chromosol* | | | *Grey chromosol* | | | *Red chromosol* | | | *Redoxic hydrosol* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pesticide | Depth (cm) | 0-2.5 | 20-30 | 30-50 | 0-2.5 | 20-30 | 30-50 | 0-2.5 | 20-30 | 30-50 | 0-2.5 | 20-30 | 30-50 |
| Diuron | Kd# | 12.1 | 18.4 | 5.2 | 27.1 | 21.8 | 51.8 | 27.3 | 15.6 | 5.4 | 39.3 | 17.6 | 11.6 |
| | Koc | 1270 | 2170 | 867 | 3390 | 2420 | 7400 | 2220 | 1320 | 635 | 5460 | 2440 | 2320 |
| | %* | 32.5 | 42.4 | 17.2 | 52 | 46.6 | 66.9 | 52.5 | 37.7 | 17.4 | 61.1 | 41.4 | 31.6 |
| Ethoprofos | Kd# | 5.8 | 4.0 | 6.3 | 12.4 | 9.9 | 12.8 | | | | 10.3 | 12.4 | 11.4 |
| | Koc | 611 | 471 | 1050 | 1550 | 1100 | 1830 | | | | 1430 | 1720 | 2280 |
| | %* | 18.8 | 13.7 | 30.2 | 33 | 28.5 | 22.9 | | | | 29 | 33.2 | 31.3 |
| Trifluralin | Kd# | 81.2 | 72.1 | 40.9 | 179 | 175 | 81.9 | 142 | 55.6 | 39.6 | 174 | 91 | 39.5 |
| | Koc | 8550 | 8480 | 6820 | 2233 | 1948 | 1170 | 1155 | 4710 | 4660 | 2414 | 1264 | 7900 |
| | %* | 76.5 | 74.2 | 62 | 87.7 | 85.7 | 76.6 | 85.1 | 69 | 64.5 | 87.4 | 78.4 | 61.2 |
| Atrazine | Kd# | 4.9 | 3.6 | 2.0 | 16.5 | 11 | 18 | 6.4 | 13.9 | 4.7 | 9.6 | 7.8 | 3.3 |
| | Koc | 516 | 424 | 333 | 2060 | 1220 | 2570 | 520 | 1180 | 553 | 2570 | 1330 | 1080 |
| | %* | 16.3 | 12.7 | 6.9 | 39.8 | 30.7 | 41.9 | 20.3 | 35.2 | 16 | 27.7 | 23.8 | 11.5 |
| Desethyl- | Kd# | 11.5 | 12.3 | 11 | 19.9 | 16.1 | 22 | 12.5 | | 13.2 | 5.7 | 24.8 | 13.5 |
| atrazine | Koc | 1210 | 1450 | 1830 | 2490 | 1790 | 3140 | 1020 | | 1550 | 792 | 3440 | 2700 |
| | %* | 31.6 | 32.9 | 29.4 | 44.3 | 39.2 | 46.9 | 33.5 | | 34.6 | 18.6 | 34.9 | 33.5 |
| Ametryn | Kd# | 8.1 | 8.3 | 4.8 | 22.2 | 15.2 | 20.1 | 8.2 | 26.6 | 5.4 | 12.9 | 9.1 | 4.4 |
| | Koc | 853 | 977 | 800 | 2780 | 1690 | 2870 | 667 | 2250 | 634 | 1730 | 1260 | 880 |
| | %* | 24.4 | 24.9 | 16.1 | 47.1 | 37.9 | 44.5 | 25 | 50.3 | 17.7 | 34 | 26.7 | 14.9 |
| Chlorpyrifos | Kd# | 114 | 71.4 | 37.1 | 341 | 175 | 149 | | | | 195 | 153 | 54.2 |
| | Koc | 12020 | 8400 | 6180 | 4270 | 1943 | 2124 | | | | 2713 | 2132 | 1084 |
| | %* | 82.1 | 74.1 | 59.7 | 93.2 | 87.1 | 85.6 | | | | 88.6 | 86 | 68.4 |
| Hexazinone | Kd# | | | | 1.5 | 2.0 | 7.2 | 13.8 | 17.2 | 6.0 | | | |
| | Koc | | | | 188 | 222 | 1030 | 1120 | 1460 | 706 | | | |
| | %* | | | | 5.7 | 7.3 | 22.3 | 35.6 | 40.7 | 19.1 | | | |

# Determined using 20g soil/L equilibrated for 30 min (2 days after pesticide application)

* Percent of pesticide remaining on sediment

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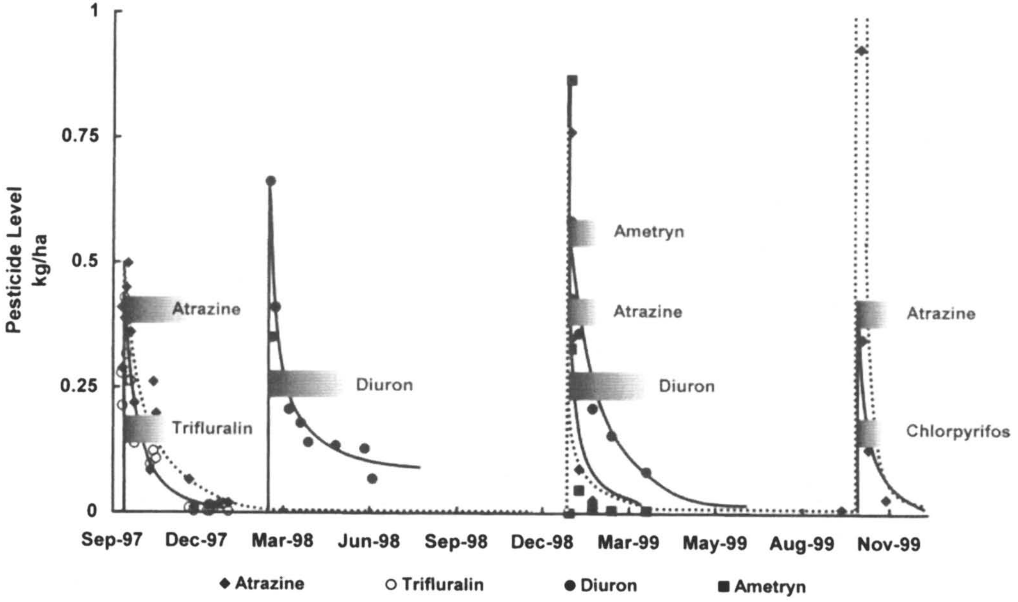

*Figure 5. Risk periods based on dissipation rates of each pesticide on redoxic hydrosol soil (0-2.5cm)*

would have produced concentrations of at least 446, 101 and 61 µg/L for atrazine, 2,4-D and chlorpyrifos respectively. Had the event occurred a week later, concentrations would have fallen to at least 44, 0.5 and 11 µg/L for atrazine, 2,4-D and chlorpyrifos respectively. Progressive changes in the Kd (Table IV) of each pesticide would also influence the relative distribution (sediment/water) in the runoff. Note that the chlorpyrifos was applied as a surface spray treatment (for army worm control), not as a controlled release insecticide (granule).

Different intensity events would produce higher mobilisation of sediment and thus produce different concentrations in runoff. Over the three 'wet' seasons on the Bundaberg site, the highest measured concentrations of atrazine in surface runoff were 670, 170 and 37 µg/L at block (4 ha), farm (45 ha) and catchment-scale (790 ha) respectively. For diuron, the maximum concentrations were 140, 66 and 11 µg/L respectively. Whilst concentrations in the runoff were quite high at times, the annual amount of pesticides exported in runoff events from this site was less than 1% of applied.

Piezometer studies on the same site showed that atrazine and diuron were entering the perched aquifer (<5 m below the soil surface), with the highest levels following the application periods. Soil moisture and pesticide residue data would suggest that rather that being leached by matrix movement to the groundwater, some of the pesticides were being moved to the perched water table by preferential flow pathways immediately after the rainfall event. However, in the more porous Mauritian soils (oxisols), where river flows are predominately driven from baseflow, some low levels of atrazine and hexazinone were detected in rivers throughout the year at multiple points throughout the catchments. In such geology and landscapes, mobile pesticides will have a greater chance of entering groundwater systems (particularly from high-rainfall areas) than in other locations where the hydrology is predominately surface-driven.

In the dry-topics study area (Atherton Tableland, North Queensland) hydrology during the wet season is mainly surface driven. In this location, the highest concentrations of atrazine and diuron in the stream draining the catchment were detected in the low-flow conditions late in the dry season when most of the herbicides were applied (65%) but by far the highest pesticide export (92%) was during the wet season. Such findings support the need for minimising pesticide applications during this high risk period – or using management practices (including other pesticides) that can reduce the risk.

## Summary of Key Findings and Related Guidelines

- In general, the breakdown or dissipation rate of pesticides under tropical sugar production was faster than previously thought.

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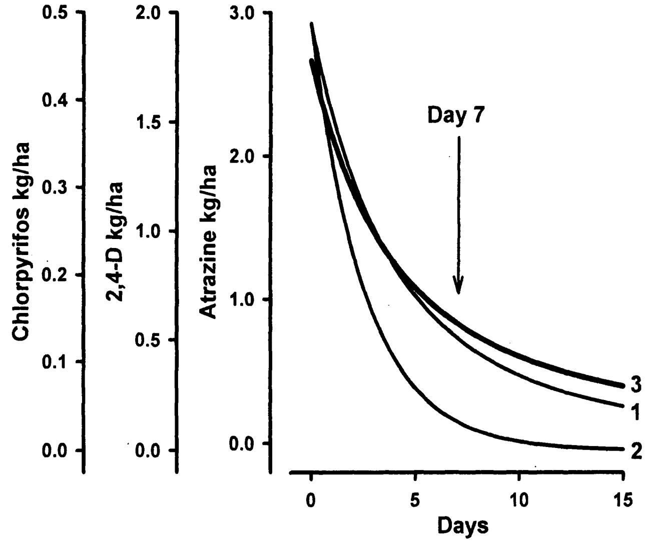

*Figure 6. Dissipation of atrazine (1), 2,4-D (2) and chlorpyrifos (3) from soil (redoxic hydrosol) surface (0-2.5cm)*

**Table IV Effect of time after application on soil adsorption (Kd*) on a redoxic hydrosol**

| *Days after application* | *Atrazine* | *Ametryne* | *Hexazinone* | *Diuron* |
|---|---|---|---|---|
| 0 | 6.8 | 6.5 | 1.1 | 19 |
| 3 | 33.8 | 52.8 | 12.5 | 67.9 |
| 7 | 38.7 | 74.1 | 15.4 | 93.6 |
| 42 | 64.4 | 130 | 24.6 | 144 |
| 56 | 76.8 | 161 | 25.7 | 210 |

*Determined using 20g soil/L equilibrated for 30 min

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

- The dissipation rate for each pesticide was variable, influenced by factors such as soil type, temperatures and management practices, but improved knowledge of dissipation rates helped define high risk periods for off-site losses.
- Annual export of pesticides (as % of applied) were low but levels of pesticides in runoff leaving cane blocks were quite high – particularly in the period soon after application.
- Improved knowledge on pesticide adsorption properties (to soils and sediments) enhanced the ability to quantify the benefits of improved sediment retention.
- There was evidence that pesticides were entering groundwater below canelands with greater mobility in the more porous soils – particularly in high rainfall areas.
- Seasonal exports of pesticides were highest during the wet season, stressing the need for additional caution in late applications.
- Increased knowledge on temporal and spatial aspects of pesticide transport processes under tropical conditions enhanced the ability to develop credible risk management models for improved management options.

## Sugarcane Industry Response

Despite a high level of cooperation, support and interest by local growers in Australia, particularly those where field studies were conducted, there was industry concern that the research may uncover unhelpful findings. Such concerns were being driven by increasing scrutiny on the sugar industry as public and government attention was building on potential threats to the Great Barrier Reef.

It is clear that in some areas, growers were quick to implement some changes as a result of new information being presented – particularly where pesticide residue data in streams was linked to their operations. This demonstrated they believed the research findings were credible and that management change was a viable option. There were cases where proposed recycling dams were 'fast-tracked' because of the new data, acting as justification for their planned investment.

Our experience indicates that there was an important role in information transfer from the involvement of agencies such as the Bureau of Sugar Experiment Stations (BSES), which provides technical support and guidance to the sugar industry and the Cooperative Research Centre for Sustainable Sugar production. The Australian Centre for International Agricultural Research (ACIAR) was instrumental in ensuring that the findings from both the Australian and Mauritian research were exposed to growers and key interest groups.

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Workshops in Australia and in Mauritius provided excellent forums for transferring detailed findings to industry and policy makers. Feedback from some environmental regulatory personnel in Australia suggested that their increased knowledge of agrochemical transport processes, combined with increased understanding of the temporal and spatial considerations, will assist their formulation of policy issues.

In Mauritius there is a close working relationship between the Mauritius Sugar Industry Research Institute (MSIRI) and sugar producers; this gave confidence that current practices were close to best practice for the major growers, but some changes could still be implemented to further minimise off-site losses of pesticides. Positive government support for this research helped to ensure the findings were positively accepted. Stronger research capabilities to address environmental contamination issues have been reinforced with the concerned public of Mauritius having been placated with the knowledge that non-point-source pollution from the sugar industry is insignificant compared with some of the point source issues which need to be addressed. The enhanced scientific capabilities at MSIRI will give some assurance to the community that as new agricultural industries emerge (e.g. edible palm plantations), that any issues of potential environmental contamination will be adequately addressed.

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## General Discussion

### Issues Regarding Interpretation and Presentation of Research Findings

For presentation of findings to industry groups, extension staff or related personnel, it is far more effective for scientists to present processed or interpreted data which have a 'carry home message'. Many scientists and pesticide analysts prefer to present raw data rather than drawing simpler conclusions in presentations. Ideally, project leaders should ensure that individual presenters are given proper guidance and support. Raw data should be made available only to those needing more detail. Research funding providers should demand that project teams have the necessary communication skills and contribute to the development of such skills by facilitating specialised training. Proper recognition of the time and resources needed for optimising interpretation and packaging of field research findings needs to be imbedded in research proposals.

### Risk Management Tools and Risk Management Concepts

Risk assessment and risk management tools are powerful approaches for assessing and managing the potential risk from agrochemical usage. A number (*14*, *15*) of such tools have been developed to help apply the scientific knowledge and understanding to land holders, catchment managers or policy makers. Kookana *et al.* (*16*) have used the 'PIRI' model for ranking the potential environmental risk of pesticides used by single or multiple industries within a catchment.

Advances in the portability, power and availability of computers in recent years has seen greater interest in the development and use of computer-based systems for information transfer and decision making. With landholders now becoming more familiar with such technology, effective risk management tools are now being developed for individual landholder use rather that at catchment scale. Simpson *et al.* (*17*) have developed such a tool (released as 'SafeGauge'), initially designed for the Australian cane industry, where site-specific details on soil type and rainfall (provided by the package) are combined with farm management information to assess the impact of proposed pesticide applications and provide management options for minimising the off-site risk.

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## Maximising Intellectual and Capital Investments of Field Research

A common frustration of many research scientists is the relatively short time frame allowed for most research projects. For some laboratory studies, short-term projects (e.g. 1-2 years) may be sufficient to complete a pilot phase. However, field research projects of only 2-3 years restricted by funding and contractual constraints have limited opportunities to deliver credible information, particular given the establishment time, the seasonal requirement of cropping cycles and the uncertainty of weather conditions. In addition, newly established field sites will often display unpredicted issues requiring refinement of original research plans or infrastructure. Whilst such changes may be positive if time was not limited, they can be highly counterproductive for short-term studies.

It is often the third and final year before there is sufficient infrastructure and reliable data to begin the complex task of holistic analysis and interpretation, a phase which ideally would be used to refine approaches and undertake further study to address data gaps as well as providing opportunity for a wider exposure to variable conditions. The pressure on the research team to deliver final reports and other products as defined in the research contract usually means that field work is terminated well before the official end of the project. An added complexity is that any temporary staff who have been employed only for the duration of the research contract, begin looking for new work – often leaving at a time when they are most needed and when their knowledge from specialist training has been maximised. Finally, field installations which are only just beginning to produce comprehensive and reliable data sets are dismantled and removed (or left in the field to deteriorate).

To ensure effective utilisation of data and the information generated, large multidisciplinary field-based research projects should cover a minimum period of 10 years to optimise the utility of the intellectual and capital investment. Serious consideration should be given to the concept of multiple 'rolling' projects which can include different mixes of funding and research teams. Obviously such a longer-term approach would need effective management and review systems in place. There is no doubt that well established multidisciplinary field-based research activities soon generate synergies that provide excellent opportunities for new innovative research activities – in a climate of enhanced knowledge and greater efficiency.

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### Some Suggestions for Field-Research Funding Providers

In recent years, there has been a shift in the policy of many research funding organisations, to move away from supporting traditional field-based research. This has been driven by their need to demonstrate that the research investment actually makes some difference, not just generating an interesting and challenging exercise for the research team. The trend is to move towards more participatory research projects where a broader level of participators are left with new awareness and knowledge and thus more likely to demonstrate 'immediate' return on the investment.

Rather than classifying traditional field-based research as too expensive, too restrictive or too academic, there is need for some funding organisations to rethink their policies and redefine their requirements for such projects, to help research teams to deliver more relevant outputs and more effective adoption of research findings. Because many technical and scientific research staff have limited awareness of effective multi-level communication and information packaging, and often see this as a role for others, serious emphasis needs to be placed on defining (and financially supporting) improved project outputs, especially multiple-level communication products.

Well planned multidisciplinary field-based research will be continue to be a vital source of factual and credible information for improved management of agrochemicals. However, there is now a greater need to ensure that such projects are not seen as isolated 'academic exercises' but are incorporated within a more holistic framework where the value of the investment can be maximised and appreciated by multiple beneficiaries.

## Acknowledgement

Thanks to Ms Lesley Ruddle of Queensland Department of Natural

Resources and Mines for technical assistance in laboratory studies and in compilation of data.

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## Chapter 22

# Minimizing Riverine Impacts of Endosulfan Used in Cotton Farming: A Science into Practice Environmental Success Story

**Nick Schofield[1], Allan Williams[2], Rachel Holloway[2], and Bruce Pyke[2]**

**[1]Land and Water Australia, Canberra, Australia**
**[2]Cotton Research and Development Corporation, Narrabri, Australia**

A major collaborative research program, Minimising the Impact of Pesticides on the Riverine Environment, responded to community and regulatory pressure about perceived widespread pollution of waterways, including incidences of fish kills attributed to the Australian cotton industry. Subsequently, research and industry knowledge has led to on-farm best practices covering all aspects of pesticide application and management. This has resulted in substantially reduced riverine contamination and a more self-confident cotton industry. The key principles that engendered these positive outcomes were: effective research collaboration between the cotton industry, research funders, research providers and regulators; high quality research on targeted questions; jointly developed solutions with cotton farmers; and a best practice framework to deliver the solutions through a self-managed industry process. This formula has resulted in one of Australia's leading examples of achieving sustainable environmental management.

In Rational Environmental Management of Agrochemicals; Kennedy, I., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

# Introduction

The Australian Cotton Industry was under public and regulatory pressure in the 1990s to manage environmental impacts of pesticide use yet there were few impact studies of broadscale pesticides application in Australia; and limited tracking of chemical use or monitoring of residual levels in the environment (1). There was a strengthening negative public image informed by the evidence of environmental impacts, especially riverine fish kills, detection of high levels of agrochemical residues in rivers and cattle fed on cotton trash or contaminated pasture and health fears of communities concerning spray drift and noise associated with aerial spraying.

The urgent need for research and development (R&D) for solutions was identified by the Cotton Research & Development Corporation, Land and Water Resources Research & Development Corporation (now Land & Water Australia) and the Murray Darling Basin Commission (hereafter called the "Funding Partners"). These agencies formed a funding partnership in 1992 and launched the program "Minimising the impact of pesticides on the riverine environment – using the cotton industry as a model" (hereafter called "the Program") (*2*).

This Program was designed to address the cotton industry issues and to lift the knowledge and capacity of Australia to manage agricultural pesticides. This description of the collaborative and research processes, summaries of some key results and evaluation evidence provides valuable insights into R&D contributing to more sustainable agricultural production.

The rapid expansion of the Australian irrigated cotton industry (Figure 1) followed construction of large dams in New South Wales and Queensland in the 1960s. Production expanded rapidly in the 1980s and 1990s to 1.5-2.9 million bales by 2005, worth between $1 billion and $1.5 billion dollars annually to the Australian economy.

Although small, growing only 3% of the world's cotton, the industry produces a significant proportion of the world's higher-quality (long staple) cotton, and exports over 95% of the crop with major buyers being Indonesia, Japan, China, Thailand and South Korea.

Cotton is prone to pests, with caterpillars *Helicoverpa armigera* and *Helicoverpa punctigera* being the more difficult to control, so spraying crops with insecticides and miticides is a major control activity and more than 30 are registered for use on cotton. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide) is regarded by farmers as a relatively 'soft' chemical on beneficial insects, with high efficacy on a range of pests. However it is known to have high aquatic toxicity (*3*) and riverine pollution attributed to spray drift and runoff from farms has been subject to numerous investigations.

An environmental audit's recommendations on improvements included: improved handling and use controls, separating new residential development and

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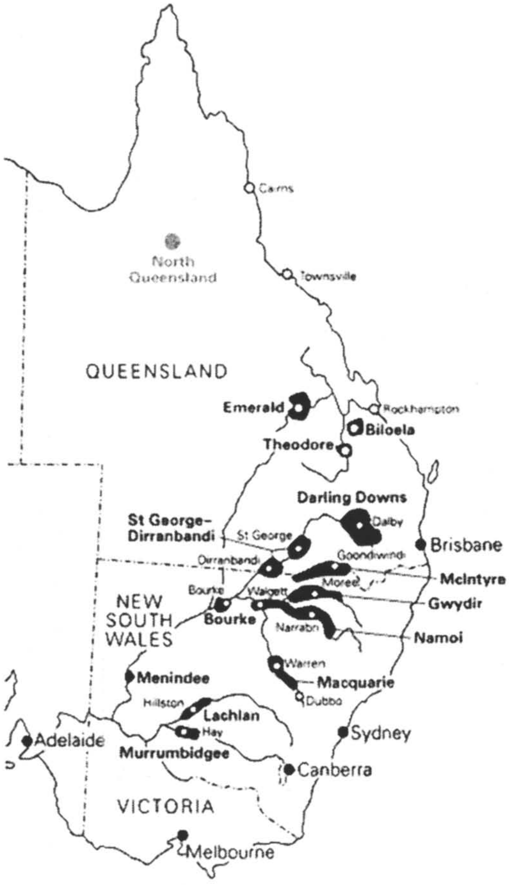
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Goondiwindi
Dirranbandi
McIntyre
Moree
Bourke
Walgett
Gwydir
NEW SOUTH WALES
Bourke
Narrabri
Namoi
Warren
Menindee
Macquarie
Dubbo
Hillston
Lachlan
Hay
Sydney
Adelaide
Murrumbidgee
Canberra
VICTORIA
Melbourne

*Figure 1. Cotton growing regions of Australia*

cotton farms, further research into integrated pest management techniques and retention on farm of all tailwater and the first flush of storm water. Public and government concern continued to grow.

## Effective Research Collaboration

The Funding Partners sought a genuine collaboration from the outset. The research funding agencies had adopted governments' philosophy for "program management" and were keen to address larger issues with partnerships and well-developed delivery mechanisms. The regulatory agencies overcame their scepticism that a research program delivering best management practice advice would significantly improve environmental performance and the New South Wales Environment Protection Agency contributed funds. On the other hand the leaders of the cotton industry were focused on industry-led management and were poised to implement research-based solutions.

The collaboration was supported by a governance structure, covering the legal agreements and management framework, delegated responsibilities and functions, program policy development, and reporting and accountability. The Funding Partners signed a contract under which project level R&D agreements could be formed and a Program Management Committee was established to allocate funds, monitor the program and appoint a Program Manager to develop and implement the Program.

A detailed review of the knowledge of pesticide impacts on the river environment fed into the program strategy (*5*). A major workshop in Goondiwindi allowed a wide range of stakeholders to work through priorities (*6*). This workshop triggered a specialist ecotoxicology workshop to identify key chemicals and organisms (*7*), and a review of the condition of rivers in cotton growing regions (*8*). These reviews contributed to the Program Management Committee setting the following objectives and outcomes:

1. To assess the impact, if any, of current pesticide use on the riverine environment.
2. To develop practical and economic methods to minimise the transport of pesticides from application sites, and to minimise their effects on the riverine environment.
3. To provide a sound scientific basis for the development of management guidelines and regulatory codes.

## Research Methodology

The research design covered all sources, transport pathways and biological impacts of pesticides, working across scales from the lab to the catchment and using concurrent monitoring of river physical, chemical and biological systems to provide integrated answers. Economic and social aspects were not formally addressed in the Program but social and economic impacts have been considered in the subsequent triple bottom line evaluation.

The research was conducted in three overlapping phases (*4*):

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Phase I: Research to determine and quantify the major pathways of pesticide movement to rivers, and to determine the level of impact of these pesticides on riverine biota. This research was to run over three years with a strong emphasis given to field-based research to understand the behaviour of pesticide application (including spray drift), deposition and dissipation from the plant canopy and soils, washoff and runoff, degradation in soil, water and plants, and transfer into water bodies. Three major field sites, two in New South Wales and one in Queensland, were established. At the same time laboratory microcosm and mesocosm dose-response studies of chemical exposure to selected organisms were commenced.

Phase II: Identification and testing of potential solutions or methods of ameliorating the problems identified in Phase I. This phase commenced after the first year of research when it had became apparent that further research was required on aerial transport mechanisms, particularly spray drift, volatilisation and vapour transport, and transport on dust particles. Additionally a greater focus was required on the transfer of chemicals between farm and river, on storm induced tailwater discharge, on chemical degradation in plants, and in understanding "real-world" modifiers of ecological impacts (e.g. sediments). The results presented here cover only endosulfan (several other pesticides were also studied) as it was the most commonly used insecticide in cotton farming in Australia and was the main contaminant of rivers in cotton growing regions (*9*).

Phase III: Incorporating research outputs into practice. The favoured approach was through best management practice (BMP), which the industry would eventually own and implement. BMP was seen as a key means of satisfying regulatory requirements.

## Spray Drift Management

Spray drift field studies were carried out over the 1993-98 cotton growing seasons in northern New South Wales (*10*). The studies monitored the off-target movement of endosulfan over a range of conditions and sought to develop effective spray drift management practices. Two methods of endosulfan aerial application were tested: (i) ultra low volume (ULV) endosulfan (through a small nozzle with 240 g/L oil-based application at 3.0 L/ha rates) and (ii) low volume (LV) or emulsifiable concentrate endosulfan (through a larger nozzle with 350 g/L water-based application with 2.1 L/ha rates). Results, normalised to a 500 m wide field, are shown in Figure 2. The ULV application had greater efficacy but suffered greater spray drift than the LV application that had less drift and lower levels beyond 500 metres but less on-leaf deposition. Resulting recommendations to managing spray drift involved using LV and spray buffers in crops so spraying only occurs to a specified distance from the crop edge determined by the environmental conditions of the day.

## On-farm Behaviour and Fate of Endosulfan

The fate of endosulfan was studied over three consecutive years on two irrigated cotton farms in NSW (*11*) and one in Queensland (*12*). Up to 70% of applied endosulfan was found to volatilize and Figure 3 shows the distribution of endosulfan residues in cotton plants at the end of the growing season. 85% of the residues were found on the leaves (*11*).

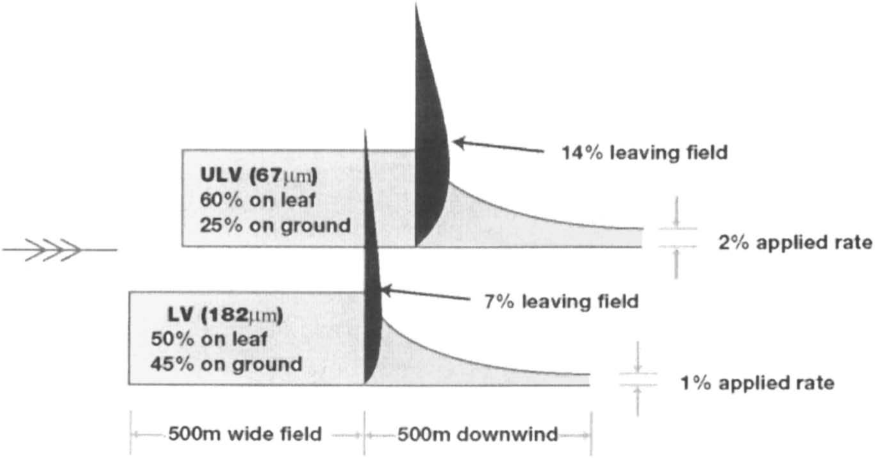

*Figure 2. Spray drift beyond a cotton field for two alternative application methods (Reproduced from reference 10. Copyright 1998.)*

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In NSW the cotton canopy cover varied from 5% at first spraying to 54% at last spraying. Endosulfan sulfate was detected almost immediately after application with its concentration increasing rapidly for the first week and then decreased steadily over an extended period. The concentration of both endosulfan isomers on plants declined extremely rapidly in the first two to three days (*11*).

Concentrations of endosulfan in foliage and stalk litter showed no clear decay pattern from the time of defoliation to six months later (*11*). Of the endosulfan reaching the soil in cotton fields, about 90% was found in the top 6 cm. Low residual levels of endosulfan (0.01 – 0.08 mg/kg) were found at the beginning of each cotton growing season and this increased to a maximum of 2 mg/kg after 2 or 3 applications. In a similar pattern to the foliage, endosulfan on soil volatilised rapidly in the first week after application. Half lives of total endosulfan were 1.6 d in foliage and 7.1 d in soil in the first rapid volatilisation phase. In the second phase, characterised by the persistence of endosulfan sulfate, half lives in foliage and soil were 9.5 d and 82 d respectively (*11*).

In Queensland about 10 applications of endosulfan were sprayed each cotton growing season to control the high pest pressure from heliothis caterpillars. On-farm measurements of total endosulfan showed: there was no build up of endosulfan in the soil from season to season; the half-life of total endosulfan was about 1 week; and repeated applications of endosulfan maintained soil residues of endosulfan above 2 mg/kg for some 2.5 months, leading to significant risk of contaminated runoff (*12*).

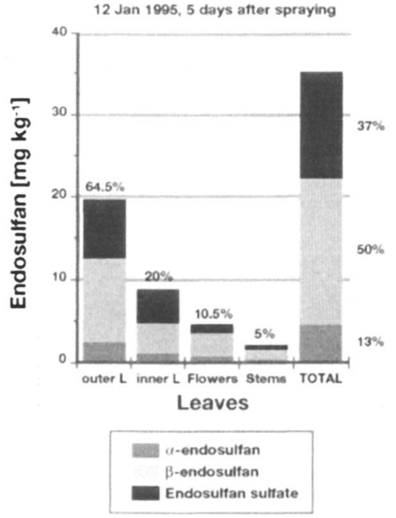

*Figure 3. Distribution of endosulfan residues in cotton plants (Reproduced from reference 11. Copyright 2001.)*

## Transport in Runoff

In NSW, analyses of irrigation and storm events showed that significant endosulfan contamination occurred for all runoff events throughout the whole cotton-growing season. The highest concentrations occurred in the early season irrigations and during large storm events. Total endosulfan load for a whole season accounted for about 2% of the pesticide applied on field (*11*). The concentration of endosulfan residues in runoff leaving cotton fields varied from 45 μg/L to 2.5 μg/L, depending on the residue levels present on the soil in the fields at the time of irrigation or storm events. The soil residue levels depend on the total amounts applied, the cotton canopy cover at application, and the time since the last application (*11*).

In Queensland, total endosulfan concentrations leaving the end of the cotton field furrows or taildrains (the drain that collects runoff from furrows at the

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lower end of the cotton field) were high (typically 8-15 µg/L) for irrigation runoff during the peak of the season (*12*) and dropped to around 2-3 µg/L towards the end of the season. Significantly higher values could be expected for major storm runoff in peak season. During a storm event in January 1996, endosulfan was traced through the irrigation area channels into the Nogoa River where concentrations of 0.5 µg/L were measured (*12*).

## Relative contributions of spray drift, vapour, dust and runoff to riverine endosulfan

Models simulating the spatial and temporal variations of endosulfan contributions to rivers included the major physical and chemical processes and, using field measurement where possible for parameter estimation and constraint, (*13*,*14*) showed that spray drift, vapour transport and runoff were all significant pathways. Transport of endosulfan on airborne dust particles was found to be insignificant. Spray drift and vapour transport both contribute low-level but nearly continuous inputs to the riverine endosulfan load during the spraying season, whereas runoff provides occasional but higher inputs (*13*,*14*).

The relative contributions attributable to the different pathways are modelled for the Namoi River in Figure 4 after 10 and 40 days at the downstream end of a 50 km stretch of the river. The 10-day contributions occur just after a major runoff event and the river concentration is dominated by the runoff component. However for most of the time, represented by 40 days after a runoff event, results show the entire riverine endosulfan concentration is due to airborne drift and vapour transport transport (*13*).

## Significance of research for management of endosulfan

The research clearly demonstrated high levels of off-site movement of endosulfan and identified the principal pathways as runoff from farms (due to irrigation and storm events) and airborne transport. Runoff contributes short (few days) but relatively high concentrations of endosulfan to rivers whereas spray drift and vapour transport contributed “constant” background inputs that result in the observed base level riverine concentrations.

The management implications were to reduce spray drift, vapour transport and farm runoff. Whilst methods were becoming available to reduce spray drift (eg spray formulations, crop buffers) and runoff (eg on-farm storm management plans, soil retention), the only way of reducing vapour transport was to reduce the total application of endosulfan. This required techniques such as integrated pest management and the strategic use of genetically modified cotton resistant to insect attack.

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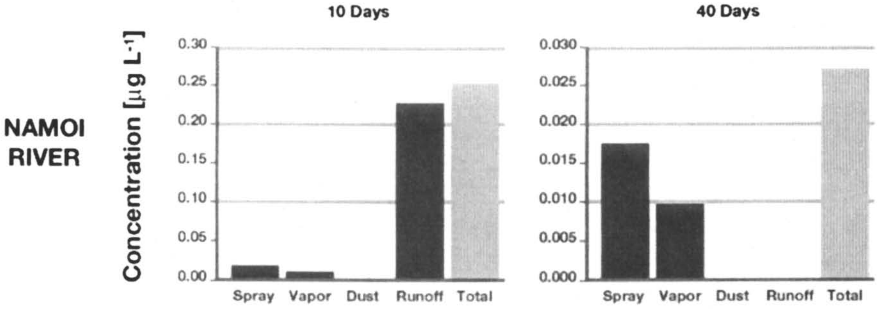

*Figure 4. Relative contributions of different transport pathways to endosulfan in rivers*

## Developing Solutions

With these clear early indications from research, the Program moved to identify solutions, recognising that cotton farmers were an important source of knowledge and would need to own the solutions if they were to be successful. Accordingly an industry and researchers facilitated workshop was organised to identify and rank potential solutions. Somewhat surprisingly, about 70% of the potential solutions were drawn from farmer knowledge (often comprising straight forward and practical actions) whilst about 30% of solutions emanated from the new research. Whilst improved practices have been developed since, the initial solutions proposed included modification of practices related to application of pesticides: improving communication between farmers; minimising the impact of pesticides on adjacent areas, the amount of pesticide applied; drift from ground rigs and aerial spray drift. The need to control soil erosion, runoff and the impact of storm events was also identified

## Implementation Through Best Management Practices

The Best Management Practices (BMP) manual contains practical environmental management guidelines within a self-assessment and prioritization process that allows cotton growers to manage off-farm transport of pesticides. The self-assessment process requires cotton growers to 'rank' their own operation in comparison with industry-recommended practices via a series of risk-rated examples. It is underpinned by the understanding of the main transport mechanisms gained through the research & development effort. These voluntary measures minimise the impacts of pesticides on the riverine environment, on community (farmers, farm workers, people in local towns) and on farm products (such as pesticide residues in beef cattle).

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The BMP committee is a sub-committee of the peak industry body, the Australian Cotton Industry Council, made up of cotton growers from both organisations in the BMP program. The Cotton Research & Development Corporation has funded the development of the BMP manual, and Cotton Australia has secured adoption of BMP by growers. The four BMP modules developed in 1996 were (i) pesticide application, (ii) storage and handling of pesticides, (iii) farm design and management, and (iv) integrated pest management. Changes were made in response to farmers and a draft manual was then subjected to an industry workshop to produce the first edition of the Australian Cotton Industry Best Management Practice Manual in 1997.

Growers developed an *action plan* for operations identified as having high risk. Gathered material, technical information, published material and legislation summarised in the BMP Manual ensured that the solutions or practices were readily available, rather than requiring further searching.

Farmers identified appropriate solutions, founded on a combination of common sense, sound science, economics and site-specific management (*17*). These are accompanied by monitoring and review procedures, again decided upon by the farmer and documented.

The BMP manual has been broadened to address other natural resource management issues relevant to cotton producers (*15*) and has become a whole farm planning tool which can deliver environmental outcomes on the farm and at a catchment scale. The BMP is also available on a single searchable CD with links from the BMP modules to more detailed technical information on a range of topics including integrated pest management, application, storage, handling spray application of pesticides, farm design and management, farm hygiene, storage and handling of petrochemicals, nutrition management soil management and land and water management, including riparian zone, vegetation and biodiversity management (*16*).

## Auditing, Monitoring and Certification of BMP

To objectively verify the on-farm implementation of best management practices on cotton farms an audit was developed and adoption surveys conducted. This formally demonstrated, particularly to regulatory agencies, that the BMPs being recommended by the industry were actually being adopted and addressing the impacts associated with applying pesticides (i.e. the BMP manual was not a 'greenwash'). The audit program was established through a commissioned pilot project in 1999, funded by the Cotton Research & Development Corporation, with a dedicated BMP audit office, under the management of Cotton Australia. The Cotton Research & Development Corporation trained 16 environmental auditors with practical experience in cotton growing to conduct BMP audits, the training course being recognised by the Quality Society of Australia and the International Environmental Auditors

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Association (*19*). The audits, which are voluntary, are designed to verify that farming operations comply with best management practices, and to identify areas where improvement is desirable.

A recent review of the BMP Program (*18*) highlighted the importance of the audit program to its external stakeholders, with over 90% of external stakeholders agreeing (and over 80% strongly agreeing) that the audit program was 'critical' to its success. There have been substantial changes in cotton farm practices since the BMP manual was introduced (*18*). For example, 97% of growers representing 99% of the cotton area surveyed had a Pesticide Application Management Plan. The reviewers also found that 94% of growers monitored weather conditions (important for spray drift minimisation), compared with only 36% five years earlier. There has also been a very high uptake of recommended integrated pest management practices, and the review found 'the cotton industry manages tail water recirculation, erosion and run-off collected from farms very well' (*18*). The review concluded that 'the majority of cotton growers have changed practices on farm for the better and would meet the current requirement to be certified as a BMP grower'(*18*).

## Monitoring the Impact of BMP in Reducing Riverine Pollution

The impact of the BMP can be detected in government monitoring of 20 riverine sites in New South Wales (*20, 21*). Riverine total endosulfan concentrations for the Gwydir River are shown in Figure 5; similar data are available for two other river systems. The trends for all three rivers are very similar. The highest recorded endosulfan concentrations were in 1991/92 immediately prior to the commencement of the Program. This was followed by a general downward trend to 2000/01, after which endosulfan has rarely been detected. That all three rivers have been well below the guideline value for endosulfan since the year 2000 is a remarkable result. Some variability in median concentrations occurred in the years 1991-2000 due to factors such as climate variability, amount of cotton planted and pest pressure. The downward trend of riverine endosulfan can be attributed to four main factors: increased awareness of cotton farmers; active implementation of best management practice on farms using the BMP Manual; steady increases in the areas planted to Bt cotton (Ingard introduced in 1996 and Bollgard II introduced in 2002) resulting in a reduction in endosulfan use; other BMP such as integrated pest management and restrictions placed on endosulfan use by the National Registration Authority. Overall this indicates that endosulfan transport by spray drift, vapour and runoff have all been effectively addressed.

A major factor in removing endosulfan from rivers has been the substantial reduction in its use (Figure 6) from 1999 to 2003. Although use increased in 2004/05 as a result of managing pest pressure, this was not associated an increase in rivers , indicating the effectiveness of the industry's BMP and the

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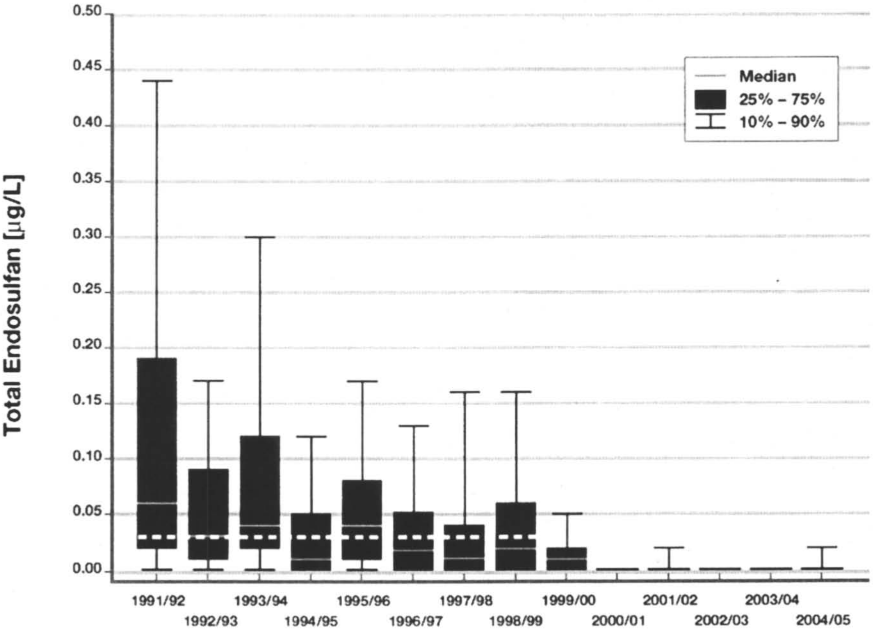

*Figure 5. Total endosulfan concentrations in the Gwydir River for the period 1991-2005 (Mawhinney pers. comm.). The broken line is the Australian and New Zealand water quality guideline trigger value for 99% ecosystem protection for total endosulfan (0.03 µg/L) (22).*

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increasing adoption of Bollgard II. Other factors have reduced the use of endosulfan including significant changes to endosulfan control in 1999-2000 with labelling that restricted total quantity of active ingredients, number of sprays, timing and method of application, establishment of buffer zones, notification of neighbours and training and record keeping.

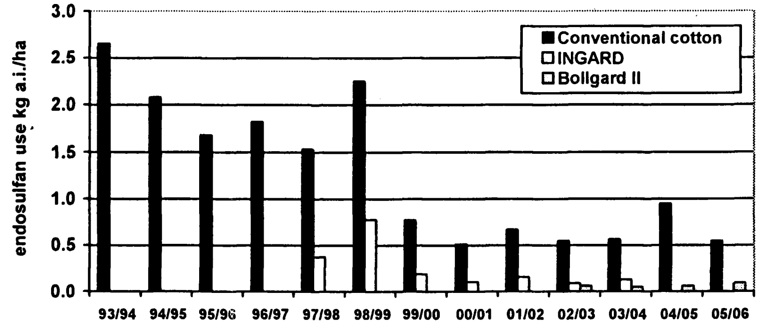

*Figure 6. Endosulfan application rates from 1993 to 2006 (Source Cotton Consultants Australia)*

## Program Return on Investment

A benefit-cost analysis for the Program and the BMP implementation was carried out (*23*) for three benefits that were considered to be both important and possible to evaluate, (i) the avoided cost of industry contraction; (ii) the avoided cost of endosulfan replacement; (iii) the benefits of anxiety reduction through improved pesticide management. The benefit:cost ratio is 5.9:1 indicating this was a very good investment.

## Wider Implications for Pesticide Management in Australia

This Program has had major spin-off benefits through the adoption of BMP approach in other industries. This 'cotton model' has been shared with rice, sugar and horticulture industries. Three products developed in this Program have broader application; the GIS decision tool "SafeGauge" (*24*) that provides management information to quantify pesticide inputs to farming systems and advice on potential environmental fate and risks (*24*); a process tool to consider factors that give the relative risk assessment for pesticides on a sub-catchment scale (*27*); and improved precision in the guidelines for Ecosystem Protection that show the 99% protection figure for important Australian species of 0.03 µg/L is strongly recommended and any relaxation from 0.03 µg/L should be assessed carefully (*28, 29, 30*).

## Conclusions

The Program has been highly successful: it has contributed to the dramatic declines in the impacts of endosulfan on riverine biota in the cotton growing regions of Australia. It has achieved this through powerful collaborations throughout the Program life. The dramatic results have re-positioned a major rural industry in Australia both in its duty of care as perceived by the wider community, and in forging a new strategic direction based on the self-implementation of best management practice. Additional important spin-offs in the development of new technologies and transfer of ideas to other rural industries have occurred.. We believe it is an example of international standing in environmental management.

The "Cotton Model" now provides an exemplary model of collaboration between government and industry and between science and practice. The key ingredients of leadership, transparency, honesty, stakeholder involvement, industry engagement, equal acceptance of scientific and farmer knowledge, and a commitment to achieving real results has demonstrated major environmental objectives can be achieved in relatively short times whilst at the same time providing a very positive triple bottom line return on investment.

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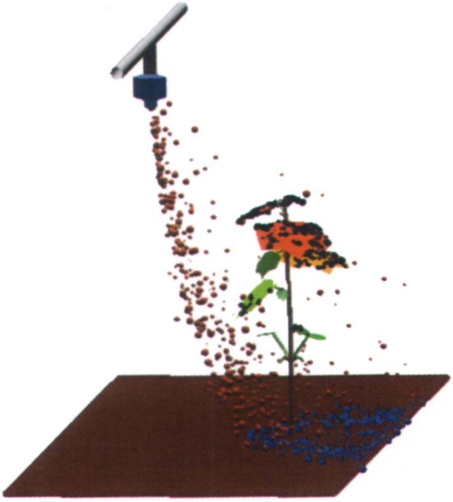

*Figure 4.1. Simulation of the movement of spray droplets and deposition on a cotton plant*

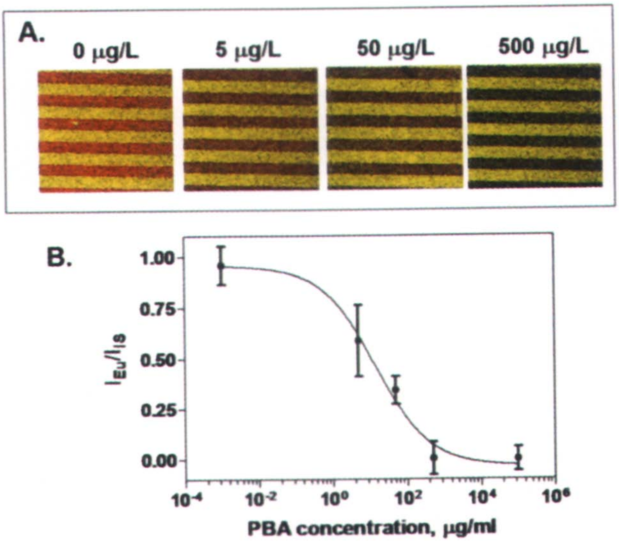

*Figure 9.4. Microimmunoassay for 3-PBA. (A) Fluorescence images of glass substrates incubated with different concentrations of 3-PBA. (B) Standard curve for the PBA microimmunoassay. (with permission from Nichkova, 2005)*

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